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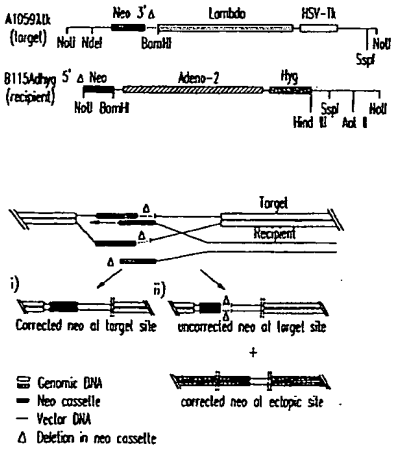

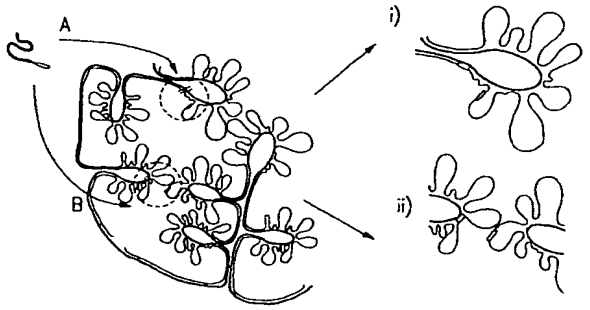

(57) Abstract

The present invention relates to a method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of: a) recombining a linear recipient vector with a target sequence present in at least one chromosome, wherein the recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to recombine with the target sequence and a tail consisting of a heterologous nucleic acid sequence; and b) identifying at least one region of the chromosome capable of recombining with the recipient vector by PCR means or by detecting a label and/or a tag on the recipient vector or the target sequence.

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A METHOD TO MAP AND ISOLATE REGIONS OF CHROMOSOMES THAT INTERACT OR ASSOCIATE FUNCTIONALLY WITHIN OR BETWEEN CHROMOSOMES *IN VIVO*

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a method for mapping and isolating regions of chromosomes that interact together or associate functionally *in vivo*. The method of the present invention is also referred to as recombination access mapping (RAM).

(b) Description of Prior Art

Currently in the field of molecular biology, it is becoming quite evident that gene regulation occurs through a complex network of processes. Transcription, replication and recombination of DNA must occur in a timely and appropriate manner or the outcome may be disastrous. Extreme control over these processes is required during development and differentiation of tissues in multicellular organisms. In contrast, disorder of these processes occurs during oncogenesis. Cancer cells often exhibit aberrant expression of genes as well as general genomic instability, a hallmark of which is an increase in recombination rates.

Activation or repression of gene expression by transcription factors or repressors respectively has been studied in great detail both *in vitro* and *in vivo*. Changes in chromatin structure are intimately linked to the activation or inactivation of a gene and can affect the replication or recombination of DNA. Mostly *in vitro* studies and limited *in vivo* data has been used to determine such changes in chromatin as they relate to DNA transcription, replication and recombination. From this data it is apparent that chromatin is organized in DNA loop domains that are organized through interaction with a nuclear protein matrix. These domains average

in size from 60-100 kb and are assumed to be flanked by matrix attachment regions (MARS). MARS have been associated with functional domains of transcription and replication. Indirect methods such as DnaseI sensitivity and DNA cleavage assays involving topoisomerase inhibitors, have provided further evidence of higher order chromatin domains which may correspond to single loops (60-100kb) and loop arrays (300kb). Functional chromatin domains seem to exist for transcription and replication (reviewed by in Jackson, D. A., *Bioessays* **17**:587-591, 1995). Strong evidence exists which suggests chromatin structure also plays a role in recombination as in VDJ recombination, formation and repair of double strand breaks in irradiated cells, and during meiosis; differences in meiotic recombination between imprinted domains.

Therefore, the central question is raised as to the interaction between chromatin domains and their accessibility to biological molecules are involved in gene regulation. Many studies of chromatin focus on *in vitro* data at the nucleosomal level (Wolffe, A. Chromatin: Structure and Function, Second Ed. *Academic Press Inc.* San Diego 1995). Unfortunately, it is most likely that large scale changes in chromatin packaging beyond the nucleosomal level are primarily responsible for the maintenance of certain chromatin states, such as early or late replication and hetero vs. euchromatin. Very few techniques exist to analyze DNA interaction *in vivo* in a global fashion over the entire genome.

Ectopic gene targeting is an alternative outcome of the gene targeting process in which a targeting vector acquires sequences from a genomic target but proceeds to integrate elsewhere in the genome. More specifically, ectopic gene targeting is a process by

which an extra chromosomal molecule (recipient) obtains DNA sequence from a target locus via one-end invasion and gene conversion followed by release of the recipient molecule and integration, complete with the newly acquired sequence from the target locus, elsewhere in the genome. Such events were first observed in gene targeting experiments involving the adenine phosphoribosyl transferase (APRT) locus in CHO cells (Adair, G. M., et al., *Proc. Natl. Acad. Sci. USA* **86**:4574-4578, 1989) and in experiments involving retroviral transfection of rat cells (Ellis, J. et al., *Mol. Cell. Biol.* **9**:1621-1627, 1989). Consequently, a model has been proposed for the mechanism of ectopic gene targeting (Belmaaza, A., et al., *Nucl. Acids Res.* **18**:6385-6391, 1990; and Belmaaza, A. et al., *Mut. Res.* **314**:199-208, 1994). Instances of ectopic gene targeting and/or ectopic gene conversion have been seen in Drosophila (roo element, p and hobo elements), plants, yeast (between dispersed repeated genes or Ty 1 repeat elements), fungi (in Ustilago maydis, chickens (Ig rearrangement)), rabbit (generation of antibody repertoire), mice (germline ectopic gene conversion in spermatids, gene conversion between Line-1 elements, and humans (gene conversion between Line-1 elements and pseudo autosomal region on X and Y chromosome).

Although the phenomenon of ectopic gene targeting is well documented, the question of where the recipient molecule integrates, with respect to the target locus, has not been determined. It is apparent from Southern analysis that the recipient integrates in most cases at a distinct site from the target but Southern analysis does not permit the determination of the relative position of the ectopic sites with regard to the target locus.

It would be highly desirable to be provided with a method allowing the identification of interactions between chromatin domains within or between chromosomes which may be involved in gene regulation. With such a method, the functional organization of the genome could be mapped. The understanding the three-dimensional (3-D) *in vivo* interactions between chromosome could help to better understand complex gene regulation during cancer or other disease states.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a method allowing to define functional organization of chromatin *in vivo* with respect to interchromosomal and interchromatin domain interactions involved in gene regulation, including but not limited to replication, transcription and recombination.

Another aim of the present invention is to provide a method allowing to mark domains of chromatin that interact functionally *in vivo* with a given gene locus for the purpose of cloning such domains or their visualization in 3-D using confocal fluorescent microscopy.

Another aim of the present invention is to provide a method allowing to define points of interaction between chromosomes involved in translocation or ectopic gene conversion within or between chromosomes.

Another aim of the present invention is to provide a method allowing to define chromatin domain interactions between chromosomes involved in epigenetic phenomenon such as imprinting, position effect variegation and transvection.

Another aim of the present invention is to provide a method allowing to produce diagnostic ectopic

gene targeting distribution profiles, such as fingerprints, for a given gene locus.

Another aim of the present invention is to provide a method allowing to determine changes in genomic organization associated with various disease states as a means of monitoring disease progression or onset.

Another aim of the present invention is to provide a method allowing to study developmental changes in multicellular organisms such as during tissue development.

Another aim of the present invention is to provide a method allowing for the placement of DNA elements or recognition sites for enzymes for the purpose of chromosomal engineering.

Another aim of the present invention is to provide a means for mapping the distribution of double strand breaks in DNA, which are natural breaks or induced by any means, over a given region of a chromosome with respect to a chromosomal DNA sequence to be studied, which in turn allows for the definition, characterization and cloning of structural and/or functional genomic domain(s) containing the chromosomal sequence being studied.

A further aim of the present invention is to provide a method allowing to assess the affects of a given drug or chemical on genomic organization and stability such as for defining oncogenic potential of a substance.

A still further aim of the present invention is to provide a method allowing to define at what time in a cell cycle chromatin domains associate functionally.

In accordance with the present invention there is provided a method hereinafter refered to as recombination access mapping (RAM), that enables the

elucidation of DNA interaction between domains of chromatin within the genome, *in vivo*.

In accordance with the present invention there is provided a method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of:

a) recombining a linear recipient vector with a target sequence present in at least one chromosome, wherein the recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to recombine with the target sequence and a tail consisting of a heterologous nucleic acid sequence; and

b) identifying at least one region of the chromosome capable of recombining with the recipient vector by PCR means or by detecting a label and/or a tag on the recipient vector or the target sequence.

In accordance with another embodiment of the present invention, there is provided a method which further comprises the step of:

c) characterizing the region identified in step b), thereby mapping the region on a chromosome or a segment thereof.

The "target sequence" may be a modified vector comprising a genomic sequence and a reporter gene or fragment thereof, which allows for the identification of the target sequence.

The "genomic sequence" may be a gene or fragment thereof.

The "target sequence" may be modified to introduce a first part of a "reporter gene", and wherein the recipient vector comprises a second part of the reporter gene, such that recombination of the

recipient vector with the target sequence allows the reporter gene to be functional, thereby allowing for the identification of the target sequence.

The "recipient vector" may further include at least one fluorescence *in situ* hybridization signal (FISH) or one radioactive *in situ* hybridization signal allowing for detection of *in situ* hybridization of the recipient vector with the target sequence.

The "recipient vector" may also include a signal *in situ* hybridization for detection by electron microscope *in situ* hybridization of the recipient vector with the target sequence.

The "tail" may have a sequence of at least 1 Kb in length, preferably of about 10 Kb in length.

The "recipient nucleic acid sequence" may be of at least 300 bp in length, preferably of at least 500 bp in length and more preferably of about 700 bp in length.

The "reporter gene" may be a selection gene.

The "selection gene" includes, without limitation, neomycin, puromycin, hygromycin and herpes simplex thymidine kinase.

In accordance with another embodiment of the present invention there is provided a method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of:

a) recombining a linear recipient vector with a target sequence present in at least one chromosome, wherein the recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to recombine with the target sequence;

b) identifying at least one region of the chromosome capable of recombining with the recipient vector by inverse polymerase chain reaction (PCR); and

c) characterizing the region identified in step b), thereby mapping the region on a chromosome or a segment thereof.

In accordance with the present invention there is also provided a method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of:

a) recombining a linear recipient vector with a genomic sequence, wherein the linear recipient vector comprises a recipient sequence of about 700 bp in length and a tail comprising a heterologous nucleic acid sequence of about 10 Kb in length and at least one fluorescence in situ hybridization signal (FISH) allowing for detection of in situ hybridization of the linear recipient vector with the genomic sequence, the genomic sequence comprises a sequence of a gene present in at least one chromosome, the recipient sequence is a nucleic acid sequence complementary to recombine with the sequence of the gene and the genomic sequence is modified to introduce a first part of a neomycin gene, the recipient vector comprises a second part of the neomycin gene, such that recombination of the recipient vector with the genomic sequence allows the neomycin gene to be functional, thereby allowing for the identification of the genomic sequence;

b) identifying a region of the chromosome to which the recipient vector is recombined by selecting surviving cells in neomycin-containing medium,

thereby indicating cells containing recombined recipient vector with the genomic sequence allowing for the expression of the neomycin gene, and detecting at least one fluorescence *in situ* hybridization signal;

c) characterizing the region identified in step b), thereby mapping the region on a chromosome or a segment thereof.

In accordance with the present invention there is also provided a method to map the distribution of double strand breaks in a chromosomal DNA sequence in order to define structurally or functionally genomic domains. The method comprises the steps of:

a) recombining a linear recipient vector with a chromosomal target sequence present in at least one chromosome, wherein the recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to the target sequence and a specific DNA sequence for polymerase chain reaction (PCR) amplification or for fluorescent *in situ* hybridization (FISH) analysis and wherein at least the recipient sequence or the target sequence contains a recognition site for an enzyme or chemical means for inducing a unique and specific double strand break within the recipient or target sequences;

b) identifying at least one region of the chromosome recombining with the recipient vector by inverse PCR or plasmid rescue;

c) characterizing the size and position of a genomic domain containing the chromosomal target and recipient sequences by at least FISH analysis or pulse field gel electrophoresis, following the formation of specific double

strand breaks within the chromosomal recipient and target sequences by enzymatic or chemical means;

d) cloning at least in part the identified genomic domain of step c); and

e) DNA sequence analysis of the genomic domain cloned in step d), thereby characterizing the genes and at least functional or structural sequence elements within the identified genomic domain.

The method of the present invention as described above may preferably be used to characterize or identify functional or structural sequence elements such as origins of replication, matrix attachment sites, transcription factor binding sites, imprinting centers or insulator elements. Of course, the double strand breaks may be have been formed *in vivo* or *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B illustrate a prefered embodiment of RAM method in accordance with the present invention.

Figs. 2A to 2D illustrate southern analysis of clones obtained and isolated with a RAM method in accordance with one embodiment of the present invention;

Fig. 3 illustrates the arrangement of the vectors sequences and their restriction enzyme sites used with a RAM method in accordance with one embodiment of the present invention to obtained the clones of Fig. 2;

Fig. 4 illustrates Fluorescent *In Situ* Hybridization (FISH) analysis of clones obtained and isolated with a RAM method in accordance with one embodiment of the present invention;

Fig. 5 illustrates a histogram of inter signal distances determined for target and recipient FISH signals in interphase nuclei; and

Fig. 6 illustrates a "Double Strand Break (DSB) Proximity" model of a RAM method in accordance with one embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a Recombination Access Mapping (RAM) method. Preferably, two vectors which can recombine to produce a functional gene such as neo+ via a one-end invasion mechanism of recombination (Adair, G. M., et al., *Proc. Natl. Acad. Sci. USA* **86**:4574-4578, 1989; Ellis, J., et al., *Mol. Cell. Biol.* **9**:1621-1627, 1989; Belmaaza, A., et al., *Nucl. Acids Res.* **18**:6385-6391, 1990; and Belmaaza, A. et al., *Mut. Res.* **314**:199-208, 1994) are used in the method of the present invention. Cells are transfected with a "target" vector. Clones containing the "target" vector are then transfected with a "recipient" vector. Ectopic gene targeting events are selected for example by G418 resistance. The distribution of ectopic events in relation to the target locus may be determined using two-color fluorescent *in situ* hybridization (FISH). Integration of the recipient may occur in close proximity to the target locus (close events: less than 2-3 Mb from the target) or in other chromosomes other than the target chromosome (far events). The distribution of ectopic integration is distinct for each locus and provides a "fingerprint" of chromatin domain interactions for a particular gene which are distinct for a given tissue, developmental stage or disease state. Interphase FISH analysis of far ectopic gene targeting events indicates that the chromatin domains containing each respective

vector can associate even though the recipient has integrated in distinct chromosome from the target chromosome.

Therefore, such domains may be identified and cloned. Cloned domains may contain important enhancers or silencers and/or other regulatory elements of gene expression. They may also contain origins of replication and/or regulatory DNA elements involved in the replication of DNA. These domains correspond to DNA elements which may inturn be used to find DNA binding proteins responsible for gene regulation and the functional structure of chromatin.

Fluorescent *in situ* hybridization (FISH) analysis, which can be used to identify the genomic location of distinct DNA sequences with a general resolution of ~100 Kb at interphase and ~2-3 Mb at metaphase (Trask, B. J., *Trend.Genet.* **7**:149-154, 1991), provides a unique tool for analysis of DNA sequences with respect to their chromosomal positions and with respect to each other.

A method for studying ectopic gene targeting that uses two vectors, a "target" and a "recipient" vector which can recombine to produce a functional gene (neo+) via the one-end invasion mechanism of recombination has been developed. A murine fibroblast cell line (LTA) was transfected with the "target" vector. Three distinct clones containing the "target" vector integrated in their genome were then transfected with the "recipient" vector. Ectopic gene targeting events, which are characterized by the acquisition of sequences from the target by the recipient vector and then its integration in the genome, were selected preferably by G418 resistance. The distribution of ectopic gene targeting events in relation to the target locus was determined using two-color FISH. The results

indicate that the distribution of ectopic gene targeting events is bimodal. Ectopic integration of the recipient vector occurred either in close proximity to the target locus (<2-3 Mb) or in altogether different chromosomes from the target chromosome. In contrast, illegitimate integration showed no bias for any single chromosome or chromosomal location at megabase resolution. A corollary to these observations is that both inter and intrachromosomal DNA interactions appear to occur during ectopic gene targeting. Therefore, the method could be used to determine which chromosomal domains within the genome are accessible to a given genetic locus.

The method of the present invention will be referred to as Recombination Access Mapping or RAM. In its most basic form, a vector comprising only minimal homology (about 300 bp) to a target locus in the genome, which may be a native DNA locus or exogenous sequences and whose location or identity may or may not be known, is utilized. This minimal recipient vector can undergo ectopic gene targeting at the target locus and upon integration, the position of the recipient vector may be mapped by inverse PCR or other oligo based technologies. Selection of ectopic gene targeting events would occur by PCR in which a band is only produced upon recombination of the target locus and the recipient vector. By this means, rapid large scale "RAM fishing" may be effected using minimal sized fragments of DNA to map *in vivo*, 3-D chromatin interactions over the entire genome.

Fig. 1A illustrates two plasmid based vectors used in the RAM method in accordance with a prefered embodiment of the invention. The vectors are depicted in linear form after digestion via Not I prior to transfection. The vector A1059λtk (target) contains a

3' truncated neo cassette. Vector B115Adhyg (recipient) contains a 5' truncated neo cassette. Both vectors contain selection markers for illegitimate integration (HSV-tk and Hyg, respectively) and specific DNA for detection via FISH analysis (lambda DNA and adenovirus-2 DNA, respectively). Fig. 1b illustrates the mechanism of ectopic gene targeting leading to reconstruction of a functional neo gene between an integrated copy of the target vector and an extra chromosomal recipient molecule. A simplified version of the vectors from panel A are depicted for clarity. One-end invasion of the of the target locus by the homologous 3' end of the recipient molecule leads to the formation of a D-loop. The invading 3' end of the recipient primes DNA sythesis leading to gene conversion and extension of the D-loop. Resolution by nicking of the D loop results in non-crossover and crossover products, the later involving integration within the target locus (i). Alternately, resolution can occur via unwinding of the newly sythesized strand and release of the recipient molecule or its displacement due to branch migration. In the case of a non-crossover event, the recipient molecule may integrate illegitimately in a different locus leaving the target locus unchanged (ii). Integration of the recipient molecule after gene conversion, elsewhere than within the target locus, is termed ectopic gene targeting. Illegitimate junctions are depicted via double, horizontal dashed lines where recipient DNA is joined to chromosomal DNA in the absence of homology.

Fig. 2 illustrates southern analysis of mother clones A1, A6 and A14 and selected daughter clones. Genomic DNA was isolated and digested before electrophoresis and capillary transfer to nylon membranes as described in materials and methods. DNA

was digested with either BamHI alone (A, B and C) or in combination with NdeI (see D) and blots were probed with a neo probe that lacked the promoter sequences (Eag I/HincII fragment of pMClneopA). Hind III digested lambda DNA was used as a marker of molecular weight and is shown in lane λ. Lane A contains the digestion profiles of mother clone A1 and five daughter clones. Two specific bands appear for the mother clone A1 (see A, lane A1 and E, A1). The two bands indicate that two target vectors are arranged head to tail in which the 5.6 kb band represents an intervector band and the 8 kb band spans the junction of one vector with genomic sequences (E, A1). These bands are maintained in 4 daughter clones (A1.2, A1.3, A1.5 and A1.9) indicating the target locus has remained intact in these clones. A1.12 is an exception in which the junction band has increased in size to approximately 8.2 kb suggesting reconstruction of the full neo gene. Digestion with NdeI in combination with BamHI should produce a 1.1 kb band for the target locus or a 1.3 kb band for a corrected neo gene (D). In the case of A1.2 and A1.5 the target locus remains uncorrected (i.e. maintenance of 1.1 bk band) while the recipient is corrected producing a 1.3 kb band. Clone A1.12, on the other hand, exhibits only a 1.3 kb band indicating that the target locus has been corrected. Such a pattern is indicative of a crossover event in A1.12 which has replaced downstream sequences from the neo gene of one vector (including the neo gene and lambda sequences of both target vectors) with sequences from the recipient (E, A1.12). In B the digestion profiles of A6 and five daughter clones are shown. Lane A6 shows a single target band of 5.0 kb which is maintained in all five daughter clones (lane A6.2 through to A6.6) and represents a single integration of

the target locus (E, A6). In C the digestion profiles of A14 and five daughter clones are shown. A single 3.8 kb band appears for A14 which indicates a single integration of the target locus in these clones (E, A14). This band is maintained in all daughter clones except for A14.4 in which the target band is shifted slightly to approximately 4.0 kb (C, A14.4). Again this would indicate that the target locus had been corrected. Indeed, double digestion with NdeI and BamHI gives the same profile as seen for A1.12. All other bands represent integrations of the recipient vector.

Fig. 3 illustrates an arrangement of vector sequences in the genome, as well as restriction enzyme sites in accordance with the method of the present invention.

Fig. 4 illustrates a FISH analysis of mother clones A1, A6 and A14 and selected daughter clones. Cell cultures were prepared for *in situ* hybridization and FISH analysis was carried out. DNA was counterstained with either propidium iodine (red, A, F and I) or DAPI (blue, B-E, G, H, J and K). Target sequences appear as green signals on a blue background or yellow on a red background. Recipient sequences appear as red signals against a blue background. Frame A shows a full complement of chromosomes from the mother clone A1 in which two copies of the target vector have been integrated into a dicentric chromosome between two centromeres (white arrow). Daughter clones A1.2 (B) and A1.5 (C) have integrated the recipient (small arrow) in close proximity to the target locus (large white arrow). Both the green and red signals can be seen separately rather than as a single white or yellow dot, suggesting the recipient and target sequences are more than 100 kb but not more than 2-3 Mb

apart (Trask, B. J., *Trend.Genet.* **7**:149-154, 1991). In clone A1.9 (D) the recipient sequences have integrated in the midarm of an acrocentric chromosome (small arrow) while the target locus has remained intact in the dicentric chromosome (large arrow). Clone A1.12 (E) shows a crossover event in which the target sequences have been completely replaced by the recipient sequences at the target locus (white arrow). Mother clone A6 is depicted in frame F in which a single target vector has integrated into small satellite arms of an acrocentric chromosome (white arrow). In both clone A6.2 and A6.3 the recipient sequences have integrated in the midarm of acrocentric chromosomes (see small arrows in G and H, respectively) leaving the target locus intact in the original acrocentric chromosome (large arrows). Frame I shows the single integration of a target vector in the mid arm of a metacentric chromosome in mother clone A14 (white arrow). The recipient sequences (small arrow) have integrated into the same metacentric chromosome within 2-3 Mb of the target locus (large arrow) in clone A14.4 (J). This is interesting as Southern analysis indicates a crossover event which should have resulted in an intermediate white signal (juxtaposition of recipient and target DNA) or loss all together of the green signal (i.e. target locus) as in A1.12 (E). Thus this clone may represent a rare event in which a crossover occured but the recipient was still able to integrate ectopically. Such a "broken arrow" suggests the commitment of both ends of the recipient molecule at the time of the recombination event rather than sequential participation of each end in recombination. Clone A14.6 is also shown in frame K, in which recipient sequences have integrated into the telomere of an acrocentric chromosome (small arrow) and the

metacentric chromosome containing the target vector (large arrows) has been duplicated, most likely by a non disjunction event.

Fig. 5 illustrates a histogram of the distribution of inter signal distances determined for target (green) and recipient (red) FISH signals in interphase nuclei. Photographic slides of nuclei were projected at distance of 3 m onto a screen and distances between red and green signals were measured in cm. The inter signal distances were pooled in bins of 0 to 12 cm (average diameter of an interphase nuclei) in 1 cm intervals. Fig. 5 depicts a histogram of the number of nuclei in each bin for random, unlinked sequences (grey bar; n=69; data from a two independent pools of ~220 clones), linked sequences (horizontal striped bar; n=63; from pooled data from 3 independent clones) and for far ectopic events (white bar; n=247; pooled data from 9 independent clones). Bin 0 represents co-localization or near co-localization of red and green signals. All values have been normalized for 63 nuclei. Black bars represent the normal distribution expected from the mean (6.37 cm) and standard deviation (3.27 cm) of the observed random, unlinked inter signal distances. The far ectopic events follow a normal distribution for bins 1-12 but the observed number of co-localizations seen (i.e. ~10 nuclei in bin 0) deviates significantly from the expected number (~1) with a p value of less than 0.0001. (note: although data were pooled for several clones to increase the total number of nuclei observed, no significant deviations were seen between independent clones with regard to the distribution of inter signal distances).

Fig. 6 illustrates a "Double Strand Break Proximity" model based on the ability of a double

strand break (DSB) to enhance recombination in the local domain in which it has occurred. Heavy lines indicate the nuclear matrix/lamina and finer lines indicate chromosomal DNA. Note that the break in chromosomal DNA at the lamina delineates the end of one chromosome and the beginning of another. When a DSB (double slanted lines on chromatin loops) occurs the cell cycle arrests and the DNA at the site of the break becomes associated with DNA repair proteins which may reside on the nuclear matrix as "repair factories". This association alters the accessibility of the domain of chromatin in which the DSB has occurred (indicated by a circle of dashed lines) such that recombination over this domain is enhanced. An incoming linear DNA molecule can mimic a DSB and may therefore be targeted to repair factories at the nuclear matrix much like genomic DSB's. Accordingly, if linear recipient DNA (dark rectangle) is used by the cell to repair a genomic DSB in the same domain of accessibility as the target locus (white rectangle), one end invasion of the target locus and gene conversion would reconstruct a functional neo+ gene (Fig. 6A). Resolution of the event by unwinding would lead to two fluorescent spots adjacent to each other, the distance between them being within a local domain of chromatin accessibility of less than 2-3 Mb (i). Cross-over would lead to deletion of intervening sequences between the target locus and the site of integration of the recipient (with only one fluorescent spot for the recipient present). Alternately, far ectopic events would involve interaction of chromatin domains of two separate chromosomes (Fig. 6B). A linear recipient molecule used to repair a DSB on one chromosome would be able to interact with the target locus on a different chromosome if the domains containing the

target locus and DSB are in close proximity to each other (most likely by association with the nuclear matrix). One end invasion and gene conversion at the target locus would lead to reconstruction of the neo+ gene. Release and unwinding of recipient would leave the target locus and chromosome unrearranged with the recipient integrating in the chromosome with the DSB (ii). Resolution by cross-over with the target locus would lead to translocation between the two chromosomes.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Ectopic Gene Targeting

Vector Construction and Preparation

Plasmid pA1059λTk (target) and pB115AdHyg (recipient) were derived from pMClneopA (Stratagene). Briefly, 3' deletion of NgOmI/BamHI fragment or 5' deletion of EagI/NdeI fragment of the neomycin resistance gene, respectively, was followed by introduction of Not I restriction site in the NdeI or Aat II restriction site (to allow linearization of the vector). Next a herpes simplex virus thymidine kinase (HSV-tk) cassette (from pAGO) and a hygromycin (hyg) cassette (from p3'SS, Stratagene), respectively, were cloned into the multicloning site 3' of the truncated neomycin gene. Finally, 16 Kb λ virus sequence (GibcoBRL) or adeno viral sequence (GibcoBRL), lacking a Not I restriction site, was cloned into the vectors between the the neomycin and HSV-tk or hygromycin cassette, respectively. Due to the large size of the vector, subsequent subcloning to prepare transfection

quantities of DNA was carried out using SURE cells (Stratagene) and normal alkaline lysis miniprep followed by G-50 column was used to purify the DNA. Vector DNA was linearized by Not I and subjected to phenol chlorophorm extraction and ethanol precipitation before being resuspended in 1 X TE for storage at -20°C.

Cell Culture and Transfection of LTA murine fibroblasts

LTA murine fibroblasts (Tk-, Aprt-) were cultured at 37°C, 5% CO_2 in complete medium (DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS)). Cells were split the day before transfection and plated at either 5 x 10^5 - 1 x 10^6 cells ($CaPO_4$) or at ~60% confluence (electroporation). Mother cells lines were produced by electroporation of LTA cells. Briefly, cells were trypsinized and concentrated by centrifugation, the cells were then resuspended in 1 ml of complete medium at room temperature. Upon counting, cells were diluted to 2.5 to 5 x 10^6 cells/ml with complete medium and 400 ul of cell suspension was electroporated (300 volts, 900 uF) using a gene Zapper 450/2500 apparatus (IBI) in the presence of 1-2 ug of target plasmid linearized at Not I. Cells were selected for integration of the plasmid in HAT medium. Positive clones were subcloned using glass cloning rings and expanded in culture for no more than 5 passages before being stored in liquid nitrogen. Genomic DNA was digested with restriction endonucleases and Southern analysis was carried out to determine the number of integrations. Cells exhibiting a simple hybridization pattern of integrated plasmid were then subjected to $CaPO_4$ transfection (Current Protocols, John Wiley Inc. USA, p. 9.1.4-9.1.9, 1996). Cells were fed 2 hours prior to transfection with 10 ml of fresh medium. Approximately 10 ug of DNA was coprecipitated

with $CaPO_4$ and the precipitate was left on the cells for 4 hours followed by 3 min DMSO shock (10% DMSO in complete medium) or 16 hours without shock. Cells were washed with PBS twice and fed with 10 ml of complete medium. Thirty six hours after transfection 400 ug/ml of G418 (GibcoBRL) was added to the medium. G418 resistant colonies were picked as described for HAT resistance above. In addition, colonies were also subjected to hygromycin (GibcoBRL) at 250 ug/ml to determine resistance to the antibiotic.

Harvest of Cells for Fluorescent *In situ* Hybridization

LTA fibroblasts were grown to near 95% confluence before being trypsinized and replated at ½ to ¼ original confluence. Depending on the growth characterisitics of each clone, harvest of cells began at 20 to 22 hours after trypsinization by the addition of 2 drops of colchemid per 5 ml. Cells were incubated at 37°C, 5% CO_2 for 2 hours in the presence of colchemid, after which the cells were trypsinized and collected by centrifugation in 15 ml falcon tubes. Cells were then subjected to hypotonic shock by the addition of 10 ml of KCl (0.07 M, Sigma) for 20 minutes at 37°C. Cells were then centrifuged again before fixation in 10 ml of ice cold Carnoy I (3 part MeOH/ 1 part acetic acid). Fixation was repeated 3-4 times and cells were dropped on frozen slides (Fisher). Slides were cured for 24 hours at room temperature before being frozen at -20°C.

Southern Analysis

Genomic DNA was prepared as described above and digested with restriction endonucleases. Digested DNA was electrophoresed on 0.7% agarose gel (Agarose-NA, Pharmacia Biotech) and transferred to nylon membranes (Hybond N, Amersham). Hybridization was carried out with radiolabelled probe in 0.5 M sodium phosphate, pH

7.2, 7% SDS and 1 mM EDTA for 16-24 hours at 65°C. The blot was washed with several changes of 40 mM sodium phosphate, pH 7.2, 0.1% SDS at 65°C, and autoradiographed at -80°C for 3 to 7 days.

Fluorescent *In situ* Hybridization

FISH analysis was carried out as previously described (Lemieux, N. et al., *Cytogenet. Cell Genet.* **59:**311-312, 1994). Briefly, after Rnase treatment, chromosomes were denatured in 70% formamide in 2 X SSC at 70°C for 2.5 minutes. Hybridization was performed overnight at 37°C in 50% formamide, 10% dextran sulphate, 2X SSC, 0.1% sodium dodecyl sulphate, 1X denhardt (0.02% polyvinylpyrolidone, 0.02% Ficoll, 0.02% BSA, pH 7), and 1 mg/ml denatured sonicated salmon sperm DNA. Probes were denatured for 10 minutes at 95°C in the same medium. Probe concentration was 4-5 ng/ul of dig-labeled adeno-2 viral DNA and/or 5-6ng/ul of biotin labeled λ virus DNA in a volume of 20 ul per slide. Rinses were performed at 37°C for 2 minutes, twice in 50% formamide in 2X SSC followed by twice in 2X SSC. In addition high stringency washes were performed at 42°C for 15 minutes once in 50% formamide in 2X SSC followed by a single 2X SSC rinse for 8 minutes at 37°C. Probes were generated from using the Bionick Labelling System (GibcoBRL) for biotin labeled probe or the Nick translation Kit (with addition of dig-11-dUTP, Boehringer Mannheim) for digoxigenin labelling.

Fluorescent Detection and Image Acquisition

After hybridization, the slides were incubated 45 minutes at 37°C with rabbit antibiotin (Enzo), 4.6 ug/ml, in PBT (PBS: 0.2 N NaH_2PO_4, 0.2 Na_2HPO_4, 0.15 M NaCl pH 7.3, containing 0.15% BSA and 0.1% Tween 20). After 2 rinses in PBT at room temperature for 5 minutes, incubation was continued for 45 minutes in the

presence of 10 ug/ml of biotinylated anti-rabbit goat antibody (GibcoBRL). Again after incubation slides were rinsed again as described before addition of fluorescein-streptavidin conjugate (GibcoBRL) at 8 ug/ml for final incubation of 45 minutes. During double detection the incubations continue with anti-dig mouse antibodies (1 ug/ml), anti-mouse-sheep Fab fragment (14 ug/ml) and anti-dig-sheep-rhodamine antibody (20 ug/ml) (Boehringer Mannheim). Counterstaining of DNA by propidium iodide (as described in Lemieux, N. et al., *Cytogenet. Cell Genet.* **59**:311-312, 1994) or DAPI or was carried out before visualizing slides in presence of 10-15 ul of antifade solution. Antifade solution contained p-phenylene-diamine (PPD, Sigma, USA), 1 mg/ml of a mixture of glycerol:PBS 9:1 (v:v) adjusted to pH 9 with NaOH. Slides were visualized on a fluorescence microscope (Aristopan, Leitz) without a signal amplification system. Red, blue and green fluorescence was observed by viewing through a triple band-pass filter (Omega Optical Inc., Vermont, USA). Images were captured using a charge coupled device (CCD) camera (Xybion Electronic Systems) and MacProbe version 2.5 (PSI) on a Quadra 840av Macintosh™ computer. Color balance adjustments and file conversion were accomplished in Adobe Photoshop™ V2.5.1. Although images were captured electronically for publication, signals could be easily seen through the microscope and slide film was taken to attest this fact.

Results

One of the approaches of the present invention was to use ectopic gene targeting to analyse chromatin accessibility and DNA interaction *in vivo*. To do so, Fluorescent *In Situ* Hybridization (FISH) analysis was used to determine the integration pattern of an

exogenous vector in three distinct cell lines each containing a target vector. These three cell lines were derived by electroporation of the murine fibroblast cell line LTA (tk- aprt-) with the target vector A1059λtk in a linear form (Fig. 1A). The target vector contains 16 kb of lambda sequence (used for FISH analysis) flanked by a 3' truncated neo gene (used for gene targeting) and a HSV-tk gene which was used for selection. Three tk+ cell lines were chosen and designated as A6 and A14, containing a single target vector, and A1 which contains two copies of the target vector (arranged head to tail). Each of the three lines was then subjected to $CaPO_4$ transfection with a recipient vector, B115Adhyg (Fig. 1A), containing a 5' truncated neo cassette with 600 bp of perfect homology with the 3' truncated neomycin gene of the target plasmid. As in the target vector the recipient vector contains unique sequences for FISH analysis (16 kb adeno-2 DNA) and a selection gene (hygromycin). Homologous recombination between the overlapping neo sequences in the two vectors will produce a functional neo gene which can be used for clonal selection in the presence of G418. The recipient vector was linearized via a Not I site, directly adjacent to the homology, to favor ectopic gene targeting events by leaving only one end of the recipient vector homologous to the target.

The usual gene targeting process involves invasion of the target by two homologous ends of the exogenous vector. Ectopic gene targeting involves invasion of the target sequence by only one homologous end of the exogenous vector, which then primes DNA synthesis leading to gene conversion (see Fig. 1B). At this point, there can be two outcomes. The recipient vector can form a homologous junction with the target at the target site while the other end of the recipient

vector forms an illegitimate junction at or near the target site (Fig. 1B(i)). In this case the target site is modified. The other possibility is that the homologous end of the recipient vector is released and the recipient vector integrates elsewhere in the genome (Fig. 1B(ii)). In this case the target site is unchanged. Thus, by determining where the released recipient molecule integrates, one can determine what other areas (domains) of the genome were accessible to the target site at the time of the recombination event. This was accomplished by FISH analysis using probes specific for the target and recipient vector, which enabled the direct analysis of the distribution of integrated recipient DNA with respect to the target.

Characterization of Mother Clones A1, A6 and A14

Mother cell lines A1, A6 and A14 were subjected to Southern analysis to determine the copy number and structure of the integrated target sequences (see Fig. 2). By probing against the neomycin resistance gene, a diagnostic band(s) was produced for the target locus. Two diagnostic bands are apparent for A1 at 5.6 Kb (inter-vector band) and 8 Kb (junction band) indicating the integration of two target vectors in a head to tail configuration (A1 in Figs. 2A and 3). A6 and A14 exhibit a single band of 5 kb and 3.8 Kb, respectively, which indicates a single integrated copy of the target for each cell line (A6 in Figs. 2B and 3, A14 in Figs. 2C and 3).

Mother cell lines A1, A6 and A14 were then subjected to single color FISH to determine the localization of the target vector. The location of the target locus in these cell lines is shown in Fig. 4. Clone A1, which has integrated 2 copies (arranged head to tail in tandem) of the target vector, contains the integrated sequences in a single site between the two

centromeres of a dicentric chromosome (Fig. 4A). A6 contains a single integration of the target vector in the short satellite arm of an acrocentric chromosome (Fig. 4F) and A14 contains a single integration of the target vector in the mid arm of a large metacentric chromosome (Fig. 4I).

These cell lines were then transfected with the recipient vector. Selection for ectopic and illegitimate integration events was carried out in medium supplemented with G418 or hygromycin (respectively). Resistant clones were counted and homologous (ectopic gene targeting) and illegitimate integration frequencies were determined. Illegitimate integration in the three cell lines was similar exhibiting a mean frequency of 5.6×10^{-3}. Although A1 had twice the copy number of A6 or A14, homologous recombination rates for all three clones were quite similar with an average frequency of 2.0×10^{-6}. This agrees with previous reports that demonstrated that copy number does not affect homologous targeting frequencies significantly in mammalian cells.

Ectopic gene targeting exhibits a bimodal distribution

Cell lines A1, A6 and A14 were transfected with the recipient vector and targeted events ($G418^R$ clones) were selected to be analysed by FISH. $G418^R$ clones were expanded with no more than 5 passages before cryopreservation and genomic DNA extraction. In the 5 daughter cell lines analyzed for A1 all but one (see Fig. 2A, A1.12) contained the 2 characteristic bands representing the target locus (i.e. 5.6 Kb and 8.0 kb band). The loss of the 5.6 Kb target band and an apparent shift of the 8.0 kb band, coupled with sensitivity to HAT medium (i.e. tk-) and FISH analysis (Fig. 4E) indicate that daughter clone A1.12 contains a crossover event. This event most likely involved the

deletion of the 3' region beyond the neomycin resistance gene of the first integrated copy of the target vector along with the entire second copy of the target vector. Replacing these sequences is the 3' region of the recipient containing the adeno-2 viral DNA and hygromycin gene (see Fig. 3, A1.12). Consequently, both the lambda viral DNA and tk genes of both copies of the target have been deleted resulting in loss of the green fluorescent signal representing the target locus, which is replaced by the red of the recipient (Fig. 4E). It is more apparent in Fig. 2D that A1.12 contains a X-over event at the target locus, as the neo 1.1 kb band disappears and is replaced by a 1.3 kb band representing a full neomycin resistance gene (clones A1.2 and A1.5 which are ectopic events are shown for comparison).

Overall, it is important to note that of greater than 30 $G418^R$ daughter clones analyzed by Southern for 5 different mother clones, only 2 were scored for the loss of the target locus. The four other daughter clones of A1 contain bona fide ectopic integrations (i.e.target locus is intact in each of them). Two of them, A1.2 and A1.5, have the recipient integrated in close proximity to the target (see Figs. 4B and 4C, respectively) and retain the 2 characteristic target bands as shown in Fig. 2A. As well, in Fig. 2D the 1.1 kb band of target locus is present as expected for both A1.2 and A1.5. The two fluorescent signals can be resolved as two closely spaced but distinct spots at mitosis thus allowing for setting the limit on the distance between the target and recipient in these clones as being less than 2-3 Mb (Trask, B. J., *Trend. Genet.* 7:149-154, 1991). Clones A1.9 and A1.3, on the other hand, had integrated the recipient vector in a different chromosome than the target (mid arm of a

small acrocentric chromosome (Fig. 4D); and mid arm of a metacentric chromosome, respectively.

All 5 of the daughter clones derived from A6, in contrast, were the result of ectopic integration that occurred in different chromosomes than the target. A single band of 5.0 Kb representing the target locus is maintained in all 5 daughter clones indicating an intact target locus (Fig. 2B). Two daughter clones of A6, A6.2 and A6.3, are shown in Figs. 4G and 4H (respectively). Daughter clones of A14 contain the single diagnostic band of 3.8 Kb, which indicates the target locus is intact in all 5 daughter clones. An intermediate distribution of ectopic events is seen with one clone exhibiting ectopic integration less than 2-3 Mb from the target, A14.4 (see Fig. 4J), and 4 others exhibiting integration of the recipient DNA molecule in other chromosomes than the one containing the target. The diagnostic band for the target locus is very intense and slightly shifted for A14.4. This shift was suggestive of a crossover event and Southern analysis using an alternate digestion (as in Fig. 2D) indicated that the target locus had been converted (appearance of 1.3 Kb band and loss of 1.1kb band) yet FISH analysis indicated both target and recipient sequences were present (i.e. as separate spots at mitosis, approximately 2-3 Mb from each other; see Fig. 4J). A cross over would be expected to produce a single red spot as in A1.12 with loss of the green signal representing the target locus, or as an intermediate white color, which would indicate juxtaposed sequences of less than 100 Kb apart. Therefore, A14.4 most likely does not involve a crossover event and represents a rare ectopic event involving an as yet undefined mechanism. Fig. 4K shows colony A14.6, an example of one of the distant ectopic

integration events with an apparent duplication of the target chromosome (most likely explained by non disjunction).

No insertion-type events were observed in our experiments. Such events were not likely since the recipient vector was linearized prior to transfection and only one end of the gene targeting vector is homologous to the target.

Thus, it appears that there are two distinct types of ectopic integration events. Those events which are in close proximity (<3 Mb) to the target (close) and those that occur in other chromosomes which do not contain the target locus (far). Integration of the recipient vector on the same chromosome at distances greater than 3 Mb from the target was not seen. This certainly does not imply that such events would not occur but it does indicate that such integration events would not be more likely than ones on distinct chromosomes.

Pooled FISH analysis of ectopic gene targeting events

Pooled FISH analysis of an additional 24 $G418^R$ clones, 12 clones for A1, 6 clones for both A6 and A14 was carried out to discern trends in the preferences for certain chromosome morphologies for far integrations. In general, far ectopic integration events occurred in morphologically different chromosomes at multiple sites in the daughter clones of A1 and A14 mother cell lines, whereas A6 contains a large number of ectopic integrations in the mid arm of acrocentric chromosomes. A summary of all 39 clones analysed by FISH analysis, singularly or in pool, is shown in Table 1.

Table 1
Summary of G418^R daughter clones analyzed by FISH[a]

					Acro				Meta			
	Total No. of Clones[b]	X over[c]	Same Chms	Diff. Chms	Total	T	C	M	Total	T	C	M
A1	17	2	14	3	1	0	0	1	2	1	0	1
A6	11	0	1	10	7	0	0	7	3	1	1	1
A14	11	1	2	9	7	1	2	4	2	1	0	1
Totals	39	3	17	22	15	0	2	5	7	1	1	2

[a]Includes all daughter clones analyzed by pool or independently.

[b]In the case of pooled clones: 10^5 cells were pooled for each clone and the resulting pool of cells was passaged twice before harvest for FISH analysis. 100 mitosis were counted and scored for chromosome morphology and location of fluorescent signal (red) for recipient in relation to target locus (green). The resulting numbers of mitosis were converted to numbers of clones by dividing the number of each morphology type by 4.2 , as on average one would expect to see 4.2 mitosis for each of the 24 clones in 100 mitosis.

[c]Cross-overs where scored when no target (green) fluorescent signal was present but had been replaced at the target locus by the recipient fluorescent signal (red).
Abbreviations: Acro=acrocentric; C=centromere; chms=chromosomes; Dicent=dicentric; M=mid arm; Meta=metacentric; T=telomere

A1 daughter clones showed a striking number of close ectopic events within 2-3 Mb of the target (14 out of 17 clones), whereas A14 and A6 showed a much smaller number of close events (2 in 11 and 1 out of 11, respectively). Two of the close events for A1 were single cross-over events, and one event for A14 had an apparent cross-over coupled with ectopic integration, which brings the total number to 3 out of 39 clones (8%). In A6, 7 out of 11 clones (63.6%) were scored for the appearance of the recipient signal in the mid arm of an acrocentric chromosome. A14 did not show any bias for any one chromosome morphology or position and, due to the few number of far ectopic events, it was not

possible to discern a trend for such events for A1. Although superficially it would appear A6 has a bias for acrocentric/mid arm localization of ectopic integration, it must be mentioned that there are 3 times more acrocentrics in the karyotype than metacentric and thus a frequency of ~64% does not indicate a statistically significant correlation for a specific chromosome morphology and position. What The analysis does indicate is that by using a pooling approach to FISH analysis one can produce a relatively large data set for analysing the distribution of ectopic gene targeting at a given locus. Our observations also lead us to suggest that far ectopic integrations can occur in more than one chromosome for a given locus. It remains to be determined if far events are random or if they occur in specific chromosomes.

The distribution of illegitimate integration at megabase resolution is stochastic

The large number of close events in A1 vs. A6 or A14 raises a question of bias for integration in the dicentric chromosome. Southern or genetic analysis cannot indicate linkage over large distances on the same chromosome. Therefore, the distribution of illegitimate integration was analysed via FISH analysis since it gives a direct estimate of the distribution of illegitimate integration at megabase resolution.

Approximately 220 or 250 hygromycin resistant (HYG+) clones were pooled for cell lines A1 and A6, respectively, and subjected to two color FISH analysis. Approximately 3000 HYG+ clones were pooled for A14 to determine if the number of clones pooled for A1 and A6 could produce a representative distribution of illegitimate events. The distribution of illegitimate integration events in relation to the target locus was scored in 200 mitosis for relative position of the

fluorescent signal on a chromosome (centromeric, telomeric or mid arm) as well as the morphology of the chromosome (acrocentric, dicentric or metacentric) containing the signal (see Table 2). The distribution of illegitimate events for all three clones was similar, indicating that illegitimate integration in these clones can not account for any differences in the pattern of ectopic gene targeting.

Table 2
Distribution of Illegitimate Integration without Ectopic Gene Targeting

Clone (pool size)	No. of Mitosis (chms)	No. of Acro chms	No. of Meta chms	No. of Dicent chms	Mid arm	Telo	Centro	Signal in Same chms	Double Signal	Triple Signal
A1 (~220)	200 (205)	97	108	0	136	43	26	2	13	1
A6 (~250)	200 (212)	134	76	2	154	30	26	0	13	0
A14 (~3000)	200 (207)	119	80	8	132	40	27	3	12	0
Totals[a] <%>	600 (624)	350 <56.1>	264 <42.3>	10 <1.6>	432 <69.2>	113 <18.1>	79 <12.7>	5[b] <0.8>	38[b] <6.3>	1[b] <0.2>

[a] Percentage of 624 chromosomes scored unless otherwise noted;

[b] Out of 600 mitosis scored

Abbreviations: Acro=acrocentric; Centro=centromere; chms=chromosomes; Meta=metacentric; Telo=telomere

The distribution seen suggests that illegitimate integration in the presence of a chromosomal target is not biased, on a megabase scale, for any given chromosome or chromosomal position (i.e. telomere, centromere, mid arm) including the target chromosome and locus, respectively. The distribution reflects the prevalence of each chromosomal position and chromosome morphology. For example, there are ~18% telomeric signals on average and ~13% centromeric, or roughly two

to one, and ~69% mid arm. This agrees well with the majority of chromosomal DNA being scored as mid arm and a count of one centromere and two telomeres per chromosome. All chromosome morphologies are represented roughly according to the proportion found in the karyotype. The karyotype is modal at a mean of 52 chromosomes and has on average 13 metacentric (or submetacentric), 37 acrocentric, 1 dicentric and 1 dot like chromosome (see Fig. 4A). Thus the chance of targeting any specific chromosome would be approximately 1 in 52 (~2%). Targeting the same 2 Mb unit of chromatin considering approximately 6000 such units in the average diploid mammalian genome would be 2 in 6000 (~0.03%). The chance of targeting an acrocentric is ~56% and for a metacentric is ~42% which is slightly skewed from the theoretical values of 71% and 25% (respectively) towards metacentrics. One explanation for this may be that metacentrics contain more DNA in general (see size difference in Fig. 4A) than acrocentrics. Considering that in normal murine karyotype all 40 chromosomes are acrocentric and similar in size it would follow that each metacentric (composed of essentially two acrocentric chromosomes after Robertsonian fusion) contains approximately twice the amount of DNA of a single acrocentric. Therefore each metacentric may provide more potential sites for integration to occur. In fact when considering this, one can create a theoretical karyotype of 65 chromosomes (i.e. 37 acrocentrics, 26 metacentric derived acrocentrics, one dicentric and one dot like chromosome). Using this karyotype the frequency for targeting a true acrocentric becomes ~57% and targeting a metacentric (i.e. actually two acrocentrics) is ~40%. These frequencies are very close to the actual observed

frequencies in our analysis for transgene integration in acrocentric and metacentric chromosomes.

The dicentric chromosome was a target for illegitimate integration 1.6% of the time which agrees well with the theoretical value of 2%. Integration in the same chromosome as the target in about 1% of mitosis scored is noted. The majority of these were very close to the target and most likely targeted events as they appear at the same frequency as expected for ectopic gene targeting events.

Interestingly, two separate signals for the recipient vector were seen in ~6% of the 600 mitosis observed for the three pools. This would suggest that in a given transfection the incoming DNA will integrate in one locus in the majority of cells (>90%). The integrated DNA may be in tandem as well as physically separated by as much as 100 kb, as at interphase separate fluorescent signals could be discerned for loci which at metaphase appeared as a single intense spot. One triple signal was seen, but this was a rare event at ~0.2%, and is most likely a double event coupled with non disjunction rather than three separate integrations. In toto, the data suggests that illegitimate integration in mammalian cells occurs primarily at a single site, stochastically, showing no bias for single chromosomes or chromosomal locations at megabase resolution.

Interphase analysis of far ectopic events

Interphase FISH analysis was conducted for 9 clones (2 derived from A1, 4 from A6 and 3 from A14) which contained far ectopic integration events on distinct chromosomes from the target. For each of the 9 clones distances between target (green) and recipient (red) sequences were measured to determine the frequency of co-localization of signals. Similar

measurements were made for pools of clones containing illegitimate integrations of the recipient, derived from cell lines A1 and A6, to provide a random or "unlinked" distribution for co-localization of red and green signals. In addition, two clones from A1 and one clone from A14, containing close ectopic integrations, were used as a "linked" control for co-localization of FISH signals.

In a total of 247 nuclei observed for the 9 far ectopic clones, 35 nuclei (~14%) exhibited coincident or nearly coincident red and green signals. In contrast, a pool of ~440 clones containing random illegitimate integration events (unlinked loci) did not exhibit co-localization of red and green signals in 69 nuclei observed. The close (linked) control exhibited 63 coincident or nearly coincident FISH signals in all 63 nuclei observed. The distribution of inter signal distances for both the random (unlinked) control and the pool of far ectopic events approximated a normal distribution with the exception of a significant deviation ($p < 0.0001$) for the number of co-localizations for the far ectopic events (see bin 0, Fig. 5). These results suggest that during interphase the site of integration of the recipient vector in far ectopic clones is found in close proximity to the target locus in a significant number of nuclei.

Discussion

Ectopic gene targeting exhibits a bimodal distribution in murine fibroblasts. The recipient DNA molecule may integrate nonrandomly within 3 Mb of the target or may integrate in other chromosomes, perhaps randomly. If indeed far events are random then integration in the target chromosome at distances beyond 3 Mb from the target locus may also occur. In our study, no far ectopic integrations where seen on

the same chromosome as the target locus, nonetheless, such events cannot statistically be excluded. A bimodal distribution was observed for 39 ectopic gene targeting events analysed for 3 independent target loci (either separately or in pools). In contrast, illegitimate integration of the recipient vector was shown to be random at the resolution of our FISH analysis. Our experiments were carried out on unsynchronized cell populations such that the effects of the cell cycle can not be addressed directly. Neverthless, the results were highly reproducible between independent experiments.

The results obtained in accordance with the present invention, lead us to suggest a model for genomic domain interactions that takes into account the observed bimodal distribution of ectopic gene targeting. FISH analysis of the integration pattern of illegitimate events indicates that at a resolution of 10-20 Mb there is no evident bias with respect to chromosomal location. In other words, integration appears random. This is the case despite the presence of the homologous genomic target. Thus, it appears that homology *per se* does not act as a determining factor in the localization of the integration site. Yet when a double strand break is introduced in genomic homologous sequences, integration occurs highly preferentially at the site of the break by homologous recombination (Rouet, P. et al., *Mol. Cel. Biol.* **14**:8096-8106, 1994). From these observation it would seem that the location of the integration site is first and foremost determined by the occurrence of a double strand break in genomic DNA and that if this DSB occurs at or near homologous sequences then the integration will most likely involve homologous recombination.

If natural DSBs occur randomly in the genome, then a DSB in any given 2 megabase unit should occur at a frequency of about 3.3×10^{-4} considering there are 6×10^{9} bases in the diploid murine genome. Since the overall frequency of illegitimate recombination for our cell lines is 6×10^{-3}, then the frequency of integration at a DSB within a 2 megabase domain containing the target should be the product of the two (3.3×10^{-4} multiplied by 6×10^{-3}), thus 2×10^{-6}. Interestingly, this is the frequency at which ectopic gene targeting is noted which is also similar to the frequency of traditional gene targeting. This similarity in frequencies has been observed previously by other groups. These frequencies are in agreement with integration involving first a DSB break which, if it occurs at or near a homologous target, will result in gene targeting. Thus, this could explain the ectopic gene targeting events that occur in close proximity to the target. According to the above reasoning, this would mean that the DSB occurred in a domain that, although situated in a distinct chromosome, was in close proximity to the domain containing the homologous target at the time of the ectopic gene targeting event. In support of a close association of far ectopic sites to the target locus at the time of recombination, a significant number of co-localizing recipient and target signals were observed by FISH in interphase nuclei of 9 separate far ectopic clones produced from mother clones A1, A6 and A14. In contrast, no co-localizations were observed for pools of random illegitimate events which exhibited a normal distibution of inter signal distances between recipient and target sequences at interphase. It is tantalizing to speculate that such domain associations may be occurring in a cell cycle specific manner linked with

either replication or transcription. A "Double Strand Break Proximity" model, presented in Fig. 6, summarizes this hypothesis.

An obvious alternative to this model would be to invoke that gene conversion between the target and the exogenous vector occurs first, followed by release of the vector and random integration in the genome via illegitimate recombination. The frequencies for each of these events do not support this hypothesis. Gene conversion between an exogenous vector and a genomic homologous target has been measured and were found to be $<10^{-6}$. Since the frequency of illegitimate integration in our assay is 6×10^{-3}, then the frequency of ectopic gene targeting, if it occurred in these two successive steps, should be the product of the frequencies of each step or 10^{-8} to 10^{-9}. This is at least two orders of magnitude lower than what is seen for the frequency of ectopic gene targeting. Of course it may be that, after the gene conversion step, the exogenous vector becomes highly potentiated for integration. Taking this possibility into account, still the site of integration should be near the target site as depicted in Fig. 5, to explain the observed bimodal distribution which is therefore not random.

In support for the concept that integration is driven by a DSB, It has been observed that illegitimate integration of an exogenous vector occurs at only one site, greater than 90% of the time (Folger, K. R. et al., *Mol. Cell. Biol.* **2**:1372-1387, 1982; Richard, M. et al., *Mol. Cell. Biol.* **14**:6689-6695, 1994). Furthermore, when there is a gene targeting event, rarely is there also in the same cell a separate illegitimate integration event even though the later occurs usually a thousand times more frequently than the former. Thus, this suggests that in most of the

cells where an integration event (homologous or illegitimate) occurs, there is only one genomic site available for integration. Recently, it has been shown that as few as one double strand break can cause p53-dependent cell cycle arrest in human embryonic fibroblasts (Huang, L. C. et al., *Proc. Natl. Acad. Sci. USA* **93**:4827-4832, 1996). $CaPO_4$ treatment of cells alone can also induce p53 cell cycle arrest (Renzing, J., et al., *Oncogene* **10**:1865-1868, 1995). Cell cycle arrest triggered by $CaPO_4$ and/or DNA damage may therefore provide a means of limiting the number of DSBs that can accumulate during a given cell cycle, thus limiting the potential number of sites of integration of exogenous DNA.

The number and types of close (targeted or crossover) events and far ectopic gene targeting events were distinct for all three loci. These trends were even more evident upon pooled analysis of an additional 24 $G418^R$ clones from A1 (12 clones), A6 (6 clones) and A14 (6 clones). Clone A1 had the highest number of close events and the least far ectopic integrations, followed by A14 and then A6 (with the most far events and only one close event). Only clone A6 showed a strong preference for a specific chromosome location and morphology, where ~64% of ectopic integrations occurred in the mid arm of acrocentric chromosomes. A1 and A14 showed weaker trends for specific chromosomes but this may only be due to the reduced number of distant ectopic integrations seen for these clones. Since the distribution of close and far events differs between loci this suggests a site specific effect on ectopic gene targeting.

It is apparent that chromatin within the nucleus is organized in a coherent manner such that gene sequences may be accessed at certain points in the cell

cycle for replication and transcription. Compartmentalization seems to occur for these processes (ex. transcription factories (Cook, P. R., *J.Cell Sci.* **108**:2927-2935, 1995); replication factories (Jackson, D. A., *Bioessays* **17**:587-591, 1990)), the factors they require (ex. splicing factors (Spector, D.L. et al., *Cold Spring Harbor Symp. Quant. Biol.* **58**:799-805, 1993)) and the chromatin involved (chromosome territories (Cremer T. et al., *Cold Spring Harbor Symp. Quant. Biol.* **58**:777-792, 1993)). The transcription and replication factories may be able to organize DNA domains from the same chromosome or from distinct chromosomes in such a way that accessibility of one domain to another may be enhanced. Using this line of reasoning, it may not be at all surprising that domains on distinct chromosomes have access to each other.

Ectopic gene conversion occurs naturally among non allelic sequences in many organisms and the mechanism of ectopic gene targeting also seems to be conserved across phila. In the present Application, it has been demonstrated that ectopic gene targeting exhibits a bimodal distribution of integration in murine cells. This indicates that both intra and interchromosomal sites are accessible to the targeting vector. Thus, the RAM method in accordance with the present invention may by used to analyse ectopic gene targeting and to determine which chromosomal domains within the genome are accessible to a given genetic locus.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and

including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of:

a) recombining a linear recipient vector with a target sequence present in at least one chromosome, wherein said recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to recombine with said target sequence and a tail consisting of a heterologous nucleic acid sequence; and

b) identifying at least one region of the chromosome capable of recombining with the recipient vector by PCR means or by detecting a label and/or a tag on the recipient vector or the target sequence.

2. The method of claim 1, further comprising the step of:

c) characterizing the region identified in step b), thereby mapping said region on a chromosome or a segment thereof.

3. The method of claim 1, wherein the target sequence is a modified vector comprising a genomic sequence and a reporter gene or fragment thereof, allowing for the identification of said target sequence.

4. The method of claim 3, wherein the genomic sequence is a gene or fragment thereof.

5. The method of claim 1, wherein the target sequence is modified to introduce a first part of a reporter gene, and wherein the recipient vector comprises a second part of the reporter gene, such that recombination of the recipient vector with the target sequence allows the reporter gene to be functional, thereby allowing for the identification of said target sequence.

6. The method of claim 1, wherein the recipient vector further comprises at least one fluorescence *in situ* hybridization signal (FISH) or one radioactive *in situ* hybridization signal allowing for detection of *in situ* hybridization of the recipient vector with the target sequence.

7. The method of claim 1, wherein the recipient vector further comprises a signal *in situ* hybridization for detection by electron microscope *in situ* hybridization of the recipient vector with the target sequence.

8. The method of claim 1, wherein the tail has a sequence of at least 1 Kb in length.

9. The method of claim 1, wherein the tail has a sequence of about 10 Kb in length.

10. The method of claim 1, wherein the recipient nucleic acid sequence is at least 300 bp in length.

11. The method of claim 1, wherein the recipient nucleic acid sequence is at least 500 bp in length.

12. The method of claim 1, wherein the recipient nucleic acid sequence is about 700 bp in length.

13. The method of claim 1, wherein the reporter gene is a selection gene.

14. The method of claim 13, wherein the selection gene is selected from the group consisting of neomycin, puromycin, hygromycin and herpes simplex thymidine kinase.

15. A method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of:

a) recombining a linear recipient vector with a target sequence present in at least one chromosome, wherein said recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to recombine with said target sequence;

b) identifying at least one region of the chromosome capable of recombining with the recipient vector by inverse polymerase chain reaction (PCR); and

c) characterizing the region identified in step b), thereby mapping said region on a chromosome or a segment thereof.

16. A method to identify regions of chromosomes that interact functionally within or between chromosomes which comprises the steps of:

a) recombining a linear recipient vector with a genomic sequence, wherein the linear recipient vector comprises a recipient sequence of about

700 bp in length and a tail comprising a heterologous nucleic acid sequence of about 10 Kb in length and at least one fluorescence in situ hybridization signal (FISH) allowing for detection of in situ hybridization of the linear recipient vector with the genomic sequence, said genomic sequence comprises a sequence of a gene present in at least one chromosome, the recipient sequence is a nucleic acid sequence complementary to recombine with said sequence of the gene and the genomic sequence is modified to introduce a first part of a neomycin gene, the recipient vector comprises a second part of the neomycin gene, such that recombination of the recipient vector with the genomic sequence allows the neomycin gene to be functional, thereby allowing for the identification of said genomic sequence;

b) identifying a region of the chromosome to which the recipient vector is recombined by selecting surviving cells in neomycin-containing medium, thereby indicating cells containing recombined recipient vector with the genomic sequence allowing for the expression of the neomycin gene, and detecting at least one fluorescence *in situ* hybridization signal;

c) characterizing the region identified in step b), thereby mapping said region on a chromosome or a segment thereof.

17. A method to map the distribution of double strand breaks in a chromosomal DNA sequence, said method comprising the steps of:

a) recombining a linear recipient vector with a chromosomal target sequence present in at least

one chromosome, wherein said recipient vector comprises at least a recipient sequence consisting of a nucleic acid sequence complementary to said target sequence and a specific DNA sequence for polymerase chain reaction (PCR) amplification or for fluorescent in situ hybridization (FISH) analysis and wherein at least the recipient sequence or the target sequence contains a recognition site for an enzyme or chemical means for inducing a unique and specific double strand break within said recipient or target sequences;

b) identifying at least one region of the chromosome recombining with the recipient vector by inverse PCR or plasmid rescue;

c) characterizing the size and position of a genomic domain containing the chromosomal target and recipient sequences by at least FISH analysis or pulse field gel electrophoresis, following the formation of specific double strand breaks within the chromosomal recipient and target sequences by enzymatic or chemical means;

d) cloning at least in part the identified genomic domain of step c); and

e) DNA sequence analysis of the genomic domain cloned in step d), thereby characterizing the genes and at least functional or structural sequence elements within the identified genomic domain.

18. The method of claim 17, wherein the functional or structural elements are selected from the group consisting of origin of replication, matrix attachment

sites, transcription factor binding sites, imprinting centers and insulator elements.

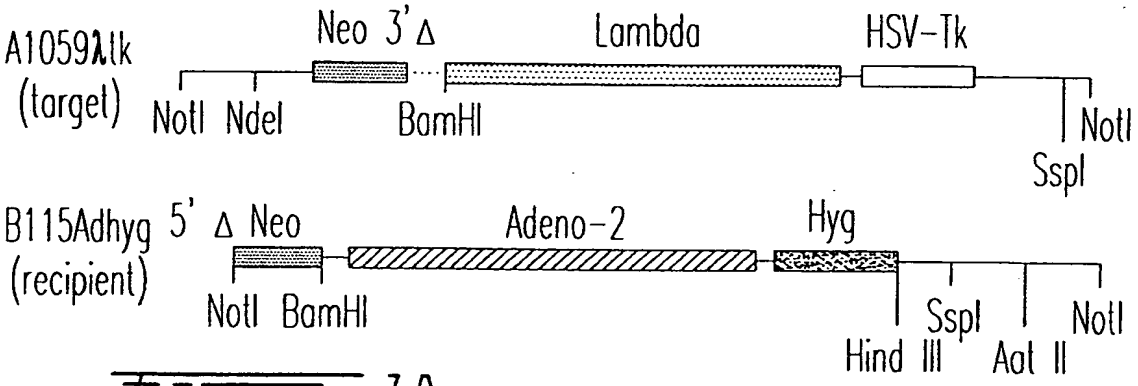
A1059λtk
(target)
Neo 3'Δ
Lambda
HSV-Tk
NotI NdeI
BamHI
NotI
SspI
B115Adhyg
(recipient)
5' Δ Neo
Adeno-2
Hyg
NotI BamHI
SspI
NotI
Hind III
Aat II

Fig - 1A

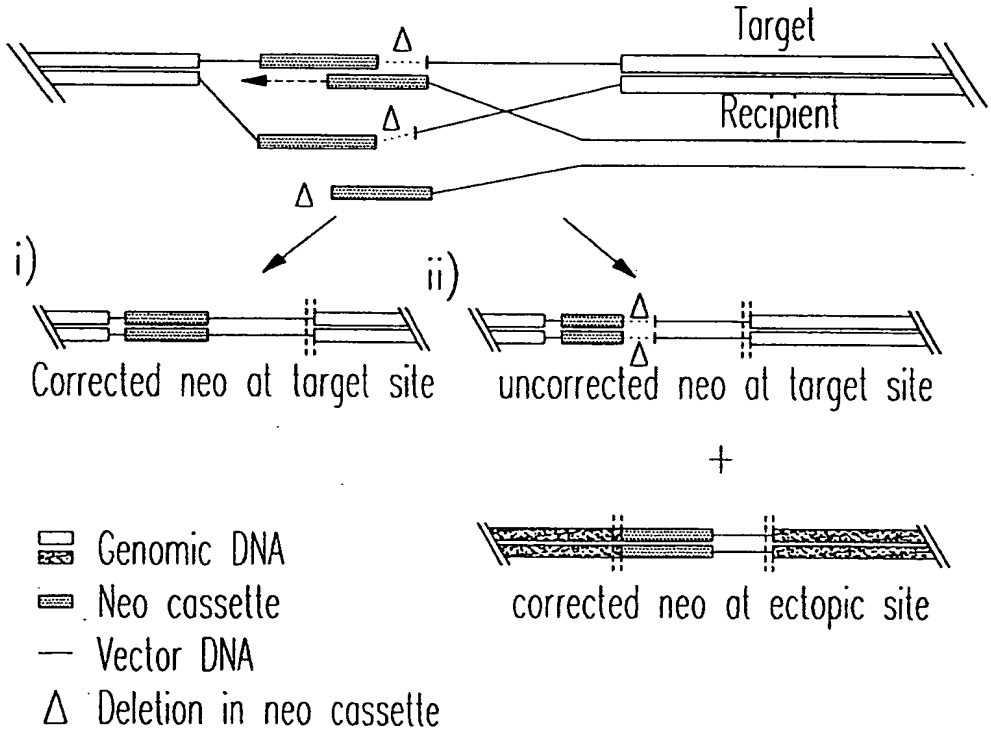
Target
Recipient
Δ
i)
ii)
Corrected neo at target site
uncorrected neo at target site
+
corrected neo at ectopic site
Genomic DNA
Neo cassette
Vector DNA
Δ Deletion in neo cassette

Fig - 1B

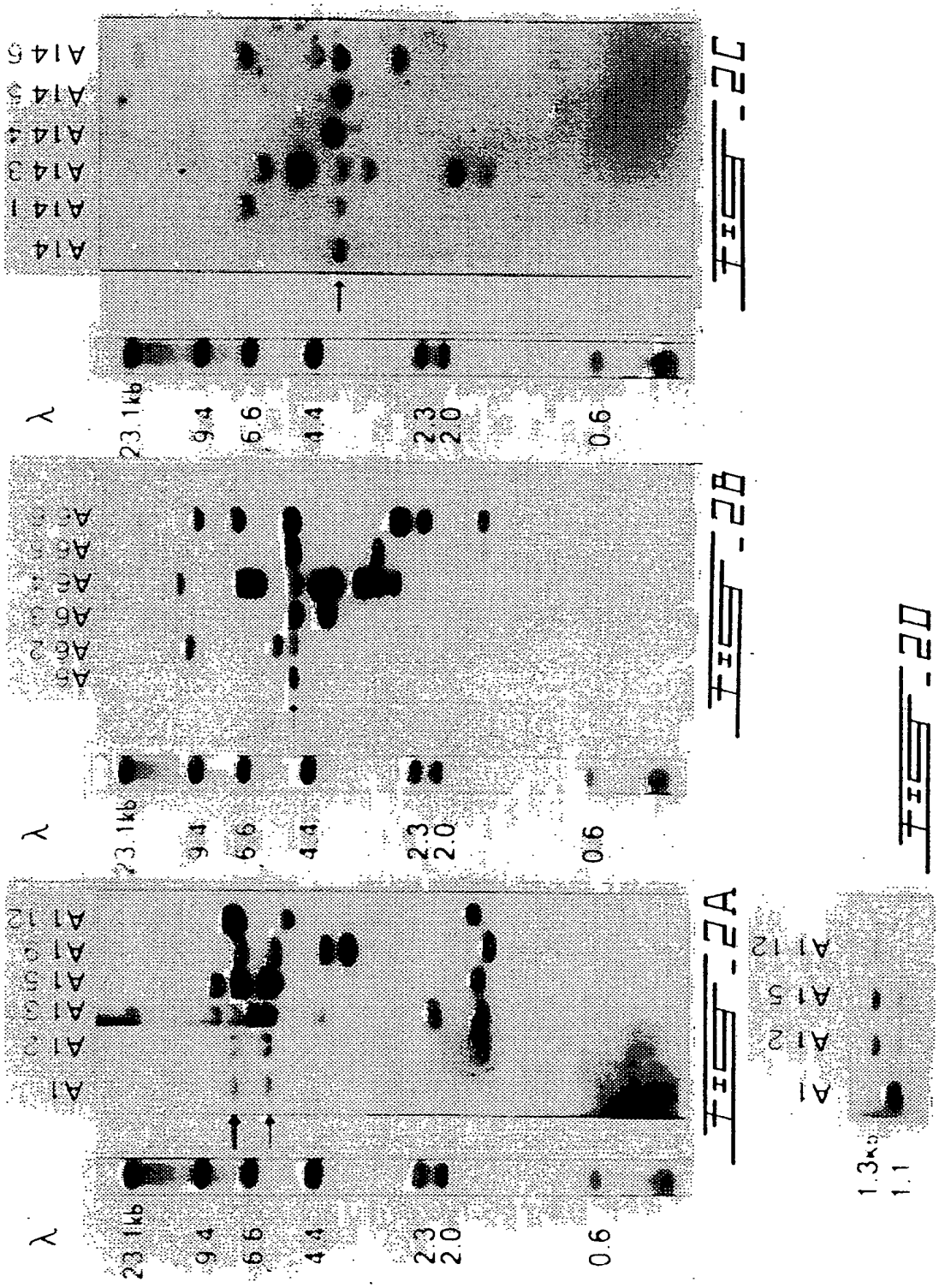
FIG-2A
FIG-2B
FIG-2C
FIG-2D
λ
23.1kb
9.4
6.6
4.4
2.3
2.0
0.6
1.3kb
1.1

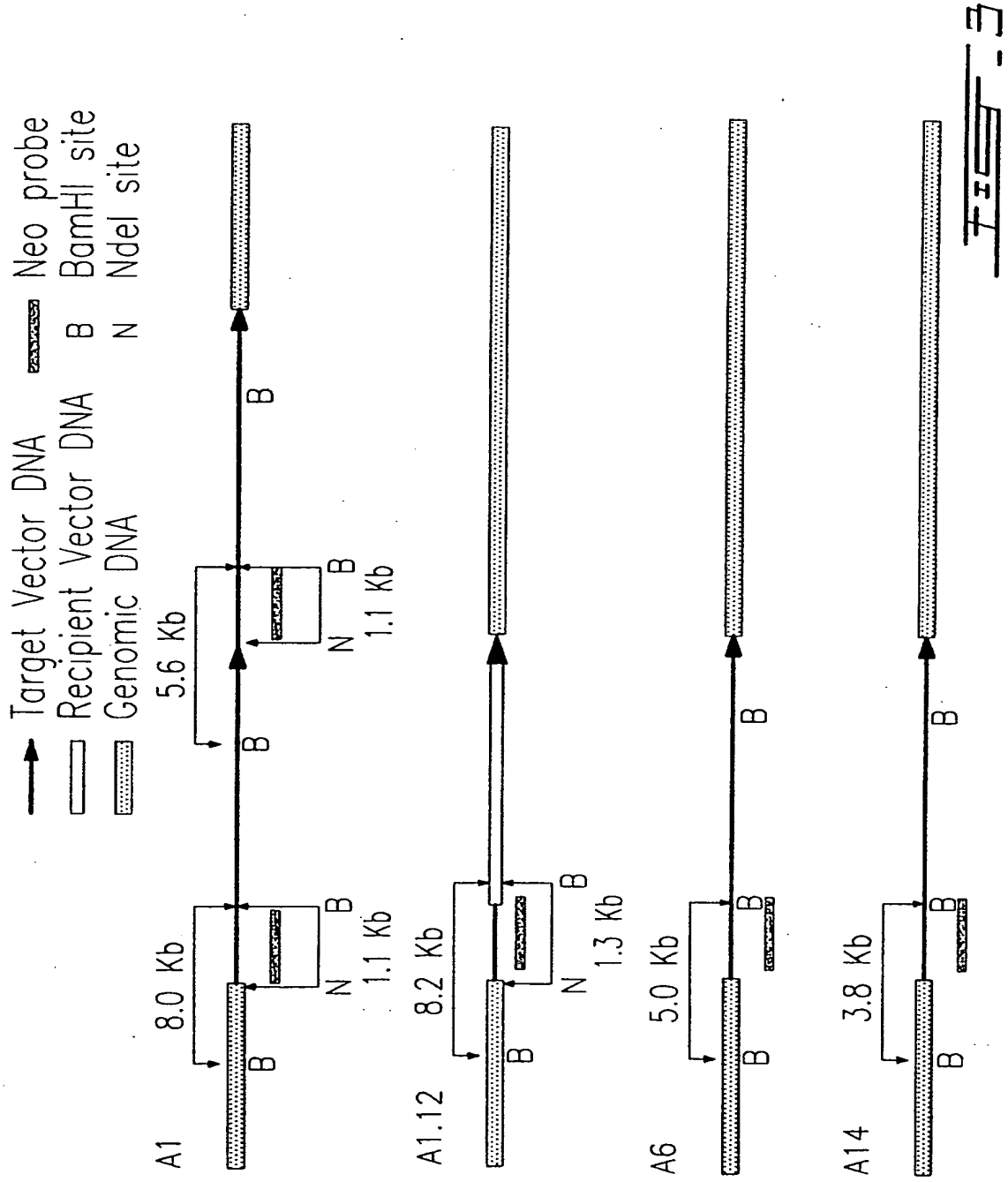
Target Vector DNA
Recipient Vector DNA
Genomic DNA
Neo probe
B BamHI site
N NdeI site
A1
8.0 Kb
5.6 Kb
1.1 Kb
A1.12
8.2 Kb
1.3 Kb
A6
5.0 Kb
A14
3.8 Kb
B
N

FIG 3

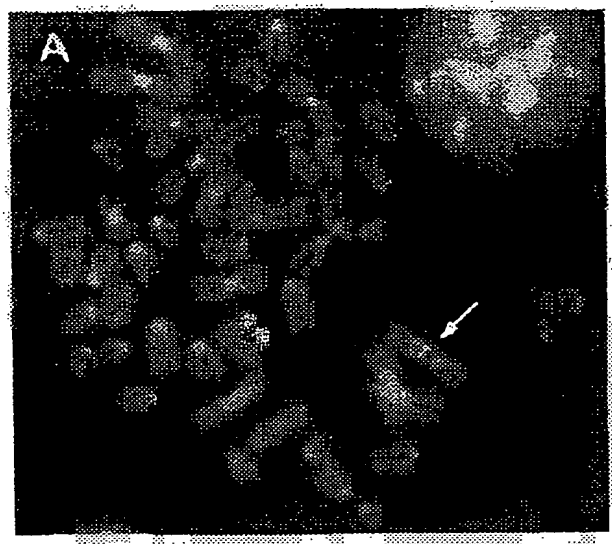

Fig. 4A

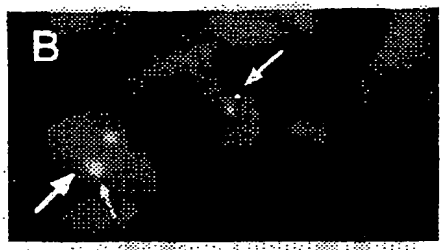

Fig. 4B

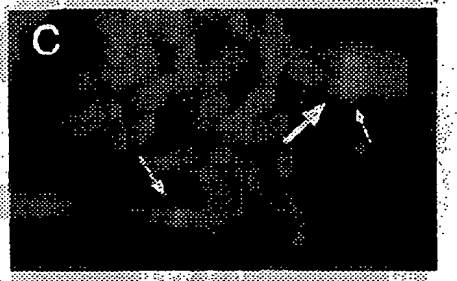

Fig. 4C

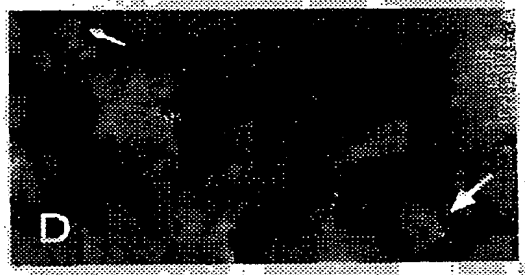

Fig. 4D

Fig. 4E

Fig. 4F

Fig. 4G

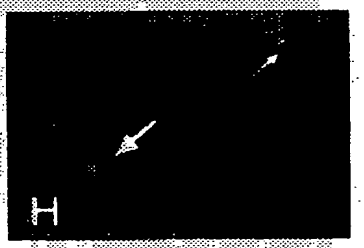

Fig. 4H

Fig. 4I

Fig. 4J

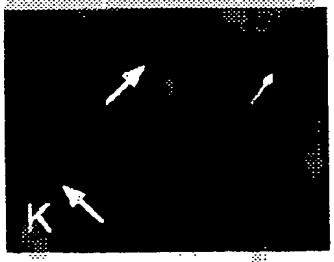

Fig. 4K

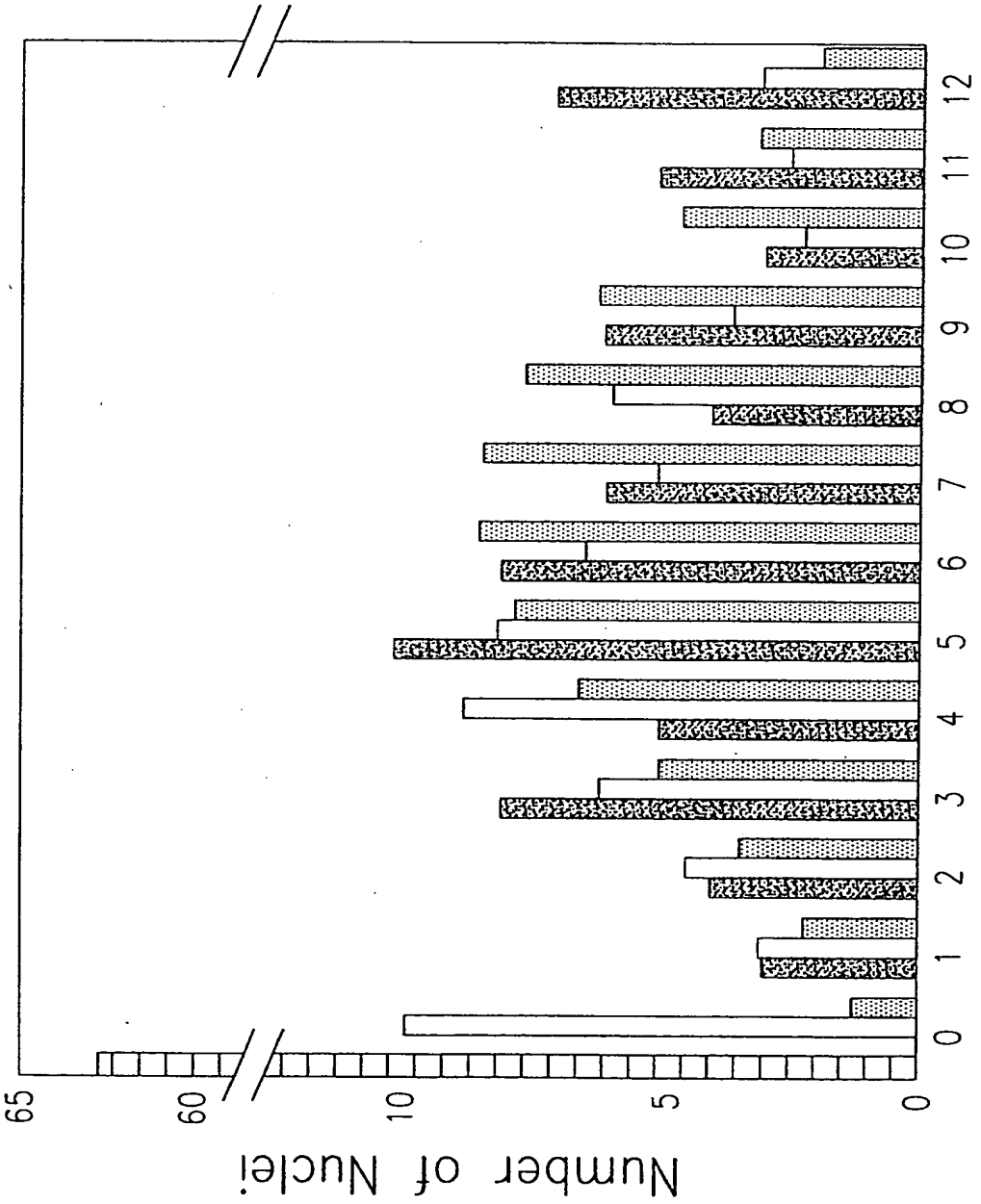

FIG. 5

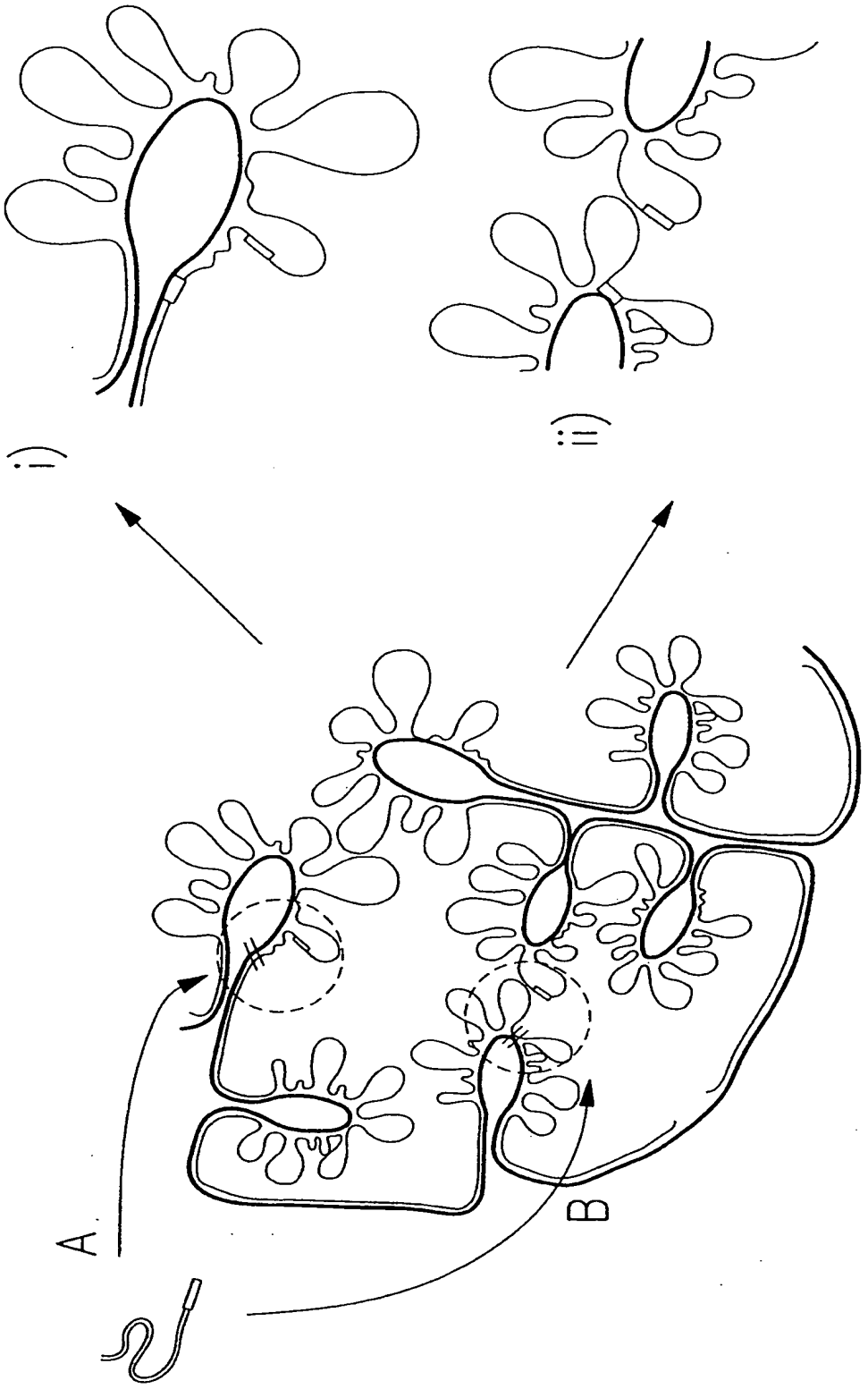

FIG - 6

INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/C /00532

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12N15/11 C12N15/90

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ELLIS J ET AL: "GENE TARGETING WITH RETROVIRAL VECTORS: RECOMBINATION BY GENE CONVERSION INTO REGIONS OF NONHOMOLOGY" MOLECULAR AND CELLULAR BIOLOGY, vol. 9, no. 4, April 1989, pages 1621-1627, XP000039605 cited in the application see the whole document	1
A	ADAIR G. M. ET AL.,: "Targeted homologous recombination at the endogenous adenine phosphoribosyltransferase locus in chinese hamster cells" PROC. NATL. ACAD. SCI. USA, vol. 86, - June 1989 pages 4574-4578, XP002077245 cited in the application see the whole document	1-18

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
11 September 1998	25/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Interr[illegible]al Application No
PCT/CA[illegible]00532

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BELMAZZA A. ET AL.,: "genetic exchange between endogenous and exogenous LINE-1 repetitive elements in mouse cells" NUCLEIC ACIDS RESEARCH, vol. 18, no. 21, - 1990 pages 6385-6391, XP002077246 see the whole document	1-18
P,X	DELLAIRE G. ET AL.,: "Ectopic gene targeting exhibits a bimodal distribution of integration in murine cells, indicating that both intra- and interchromosomal sites are accessible to the targeting vector" MOL. CELL. BIOLOGY, vol. 17, no. 9, - September 1997 pages 5571-5580, XP002077247 see the whole document	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

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(19) 世界知的所有権機関
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2001年8月2日 (02.08.2001)

PCT

(10) 国際公開番号
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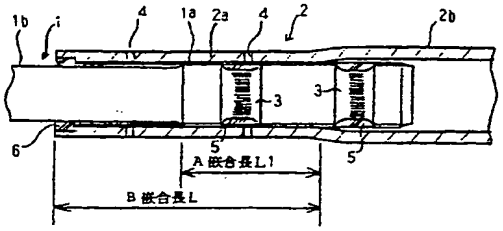
(81) 指定国 (国内): DE, GB, US.

添付公開書類:
— 国際調査報告書

２文字コード及び他の略語については、定期発行される各PCTガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: CONNECTION STRUCTURE OF EXTENDABLE SHAFT

(54) 発明の名称: 伸縮自在シャフトの結合構造



A...FITTED LENGTH L1
B...FITTED LENGTH L

(57) Abstract: A connection structure of extendable shaft, wherein resin is filled in recessed grooves (3) formed in a male spline-fitted part (1a) of an inner shaft (1) through filling holes (4) formed in a female spline-fitted part (2a) of an outer shaft (2) so as to form resin sliding parts (5) at these fitted parts (1a) and (2a) of these both shafts (1) and (2), and a resin ring (6) is installed on the inner peripheral surface of the outer shaft (2) at the tip of the female spline-fitted part (2a), whereby the tip part of the outer shaft (2) can be moved smoothly forward of a vehicle because the resin ring (6) slides on the outer peripheral surface of the small diameter part (1b) of the inner shaft (1) even if the tip part of the outer shaft (2) comes off from of the male spline-fitted part (1a) of the inner shaft (1) at the time of collapse by a secondary collision.

[続葉有]

WO 01/55610 A1

明　細　書

伸縮自在シャフトの結合構造

発明の属する技術分野

本発明は、自動車のステアリング装置等に用いる伸縮自在シャフトの結合構造に関し、詳しくは、二次衝突のコラプス時におけるアウターシャフトの車両前方への移動性を向上した伸縮自在シャフトの結合構造に関する。

背景技術

自動車のステアリング装置においては、二次衝突時に、ステアリングシャフトの一部をコラプスして収縮させることにより、運転者の保護を図っている。ステアリングシャフトの前方側の中実のインナーシャフトと、これの後方側の中空のアウターシャフトとをスプライン（またはセレーション）嵌合し、二次衝突時に、この両者の嵌合部をコラプスして、インナーシャフトをアウターシャフト内に収納し、ステアリングシャフトを収縮するようになっている。

例えば、特開平２－２８６４６８号公報および特開平１０－４５００６号公報では、両シャフトのスプライン嵌合部に、所定のクリアランスを設けて、両シャフトの軸方向の摺動性を良好に維持する一方、インナーシャフトに形成した凹溝に樹脂をインジェクション充填し、両シャフトのスプライン嵌合部に樹脂摺動部を形成して、シャフトの周方向の「ガタ」を防止すると共に、二次衝突のコラプス時に両シャフトが安定して収縮できるようにしている。

具体的には、図４に示すように、ステアリングシャフトの前方側の中

フト２の先端部は、インナーシャフト１の雄スプライン嵌合部１ａからさらに外れて、小径部１ｂの外周囲に位置するようになる。

この時、例えば、曲げ荷重がアウターシャフト２に作用すると、アウターシャフト２の先端部は、インナーシャフト１の小径部１ｂの外周面に接触するといったことがあり、その結果、アウターシャフト２の車両前方への移動が必ずしもスムーズでないといったことがある。

本発明は、上述したような事情に鑑みてなされたものであって、二次衝突のコラプス時におけるアウターシャフトの車両前方への移動性を向上した伸縮自在シャフトの結合構造を提供することを目的とする。

発明の開示

本発明に係る伸縮自在シャフトの結合構造は、インナーシャフトの嵌合部に、アウターシャフトの嵌合部を軸方向に伸縮自在に且つ回転不能に嵌合し、

前記インナーシャフトの嵌合部に形成した凹溝に、前記アウターシャフトの嵌合部に形成した充填孔を介して樹脂を充填して、これら両シャフトの嵌合部内に樹脂摺動部を形成した伸縮自在シャフトの結合構造において、

前記アウターシャフトの嵌合部の先端の内周面に、低摩擦部材を装着したことを特徴とする。

このように、本発明によれば、アウターシャフトの嵌合部の先端の内周面に、低摩擦部材が装着してあるため、二次衝突のコラプス時に、アウターシャフトが車両前方に移動して、両シャフトの嵌合部における「嵌合長」が短くなり、アウターシャフトの先端部が、インナーシャフトの嵌合部から外れて、インナーシャフトの小径部の外周囲に位置している時に、曲げ荷重がアウターシャフトに作用したとしても、アウター

【図５】

図４に示した従来の車両用ステアリングシャフトの二次衝突時の作用図である。

【図６】

図４に示した従来の車両用ステアリングシャフトの二次衝突時の作用図であって、さらにコラプスが進行した場合を示す。

発明を実施するための最良の形態

本発明の実施の形態に係る伸縮自在シャフトの結合構造を図面を参照しつつ説明する。

（第１実施の形態）

図１は、本発明の第１実施の形態に係る伸縮自在シャフトの結合構造を適用した車両用ステアリングシャフトの縦断面図である。図２は、図１に示した車両用ステアリングシャフトの二次衝突時の作用図である。

図１に示すように、ステアリングシャフトの前方側の中実のインナーシャフト１と、これの後方側の中空のアウターシャフト２とがスプライン（またはセレーション）嵌合してある。インナーシャフト１は、雄スプライン嵌合部１ａと、これより若干小径に形成した小径部１ｂとからなり、アウターシャフト２は、雌スプライン嵌合部２ａと、これより若干大径に形成した大径部２ｂとからなる。両シャフト１，２のスプライン嵌合部１ａ，２ａには、所定のクリアランスが設けてあり、これにより、両シャフト１，２の軸方向の摺動性を良好に維持している。

インナーシャフト１の雄スプライン嵌合部１ａには、全周にわたる２個の凹溝３が形成してあり、アウターシャフト２の雌スプライン嵌合部２ａには、これら凹溝３に対応して、樹脂をインジェクション充填するための複数個の充填孔４が形成してある。これにより、充填孔４を介し

ト１の小径部１ｂの外周面を滑るため、アウターシャフト２の先端部は、車両前方にスムーズに移動することができ、従来に比べて、このアウターシャフト２の車両前方への移動性を向上することができる。

また、図２に示すように、両シャフト１，２のスプライン嵌合部１ａ，２ａの「嵌合長Ｌ１」は、名目的には短くなるが、樹脂製のリング６がインナーシャフト１の小径部１ｂの外周面を滑ることを考慮すると、実質的には、比較的長い当初の「嵌合長Ｌ」を確保することができ、上記のように、アウターシャフト２は、車両前方にスムーズに移動することができる。

なお、図１に仮想線（二点鎖線）で示すように、アウターシャフト２の雌スプライン嵌合部２ａを「嵌合長Ｌ」以上に長くしておけば、コラプスが進行するに従い、「嵌合長Ｌ」を増大させることも可能である。

（第２実施の形態）

図３は、本発明の第２実施の形態に係る伸縮自在シャフトの結合構造を適用した車両用ステアリングシャフトの縦断面図である。

本第２実施の形態では、インナーシャフト１の雄スプライン嵌合部１ａには、周方向の一部にのみ部分的に形成した２個の凹溝７が設けてある。また、アウターシャフト２の雌スプライン嵌合部２ａには、樹脂を注入するための２個の注入孔８と、樹脂を吐出するための２個の吐出孔９とが形成してある。これにより、樹脂インジェクションの充填時には、注入孔８を介して樹脂を部分的な凹溝７に注入し、樹脂が溢れて余分になった場合には、吐出孔９を介して余分な樹脂を吐出して、凹溝７に、樹脂摺動部１０を形成している。

このように、雄スプライン嵌合部１ａに、周方向の一部にのみ部分的な凹溝７が形成してあるため、充填された樹脂が両嵌合部１ａ，２ａの全周にわたって必要以上に拡がることがなく、樹脂摺動部１０による摺

請　求　の　範　囲

1．インナーシャフトの嵌合部に、アウターシャフトの嵌合部を軸方向に伸縮自在に且つ回転不能に嵌合し、

前記インナーシャフトの嵌合部に形成した凹溝に、前記アウターシャフトの嵌合部に形成した充填孔を介して樹脂を充填して、これら両シャフトの嵌合部内に樹脂摺動部を形成した伸縮自在シャフトの結合構造において、

前記アウターシャフトの嵌合部の先端の内周面に、低摩擦部材を装着したことを特徴とする伸縮自在シャフトの結合構造。

2．前記低摩擦部材は樹脂製リングから成ることを特徴とする請求項1に記載の結合構造。

図 1

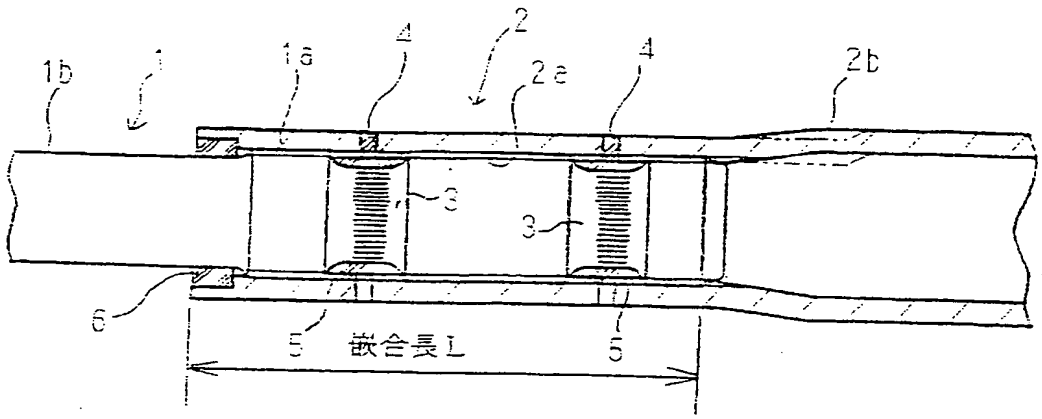


図 2

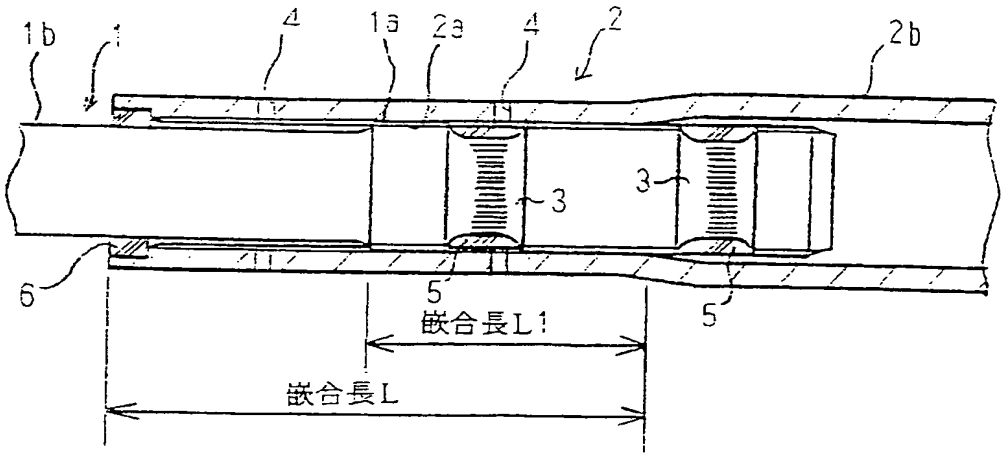


図 3

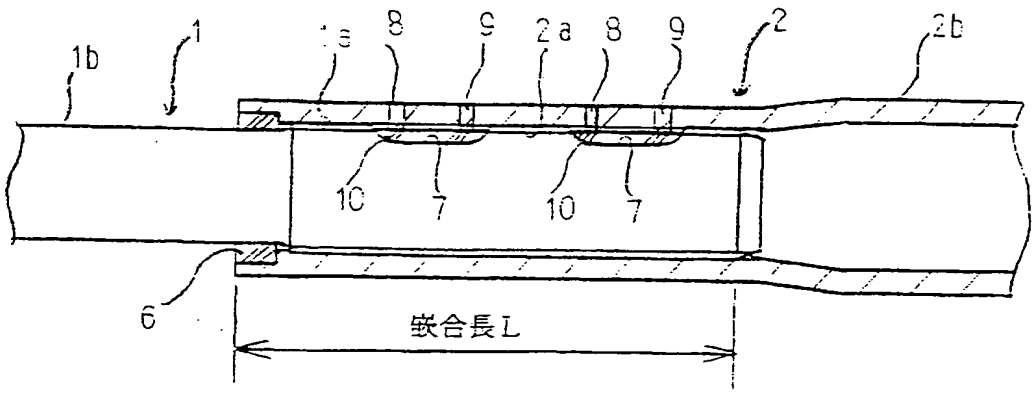


図 4

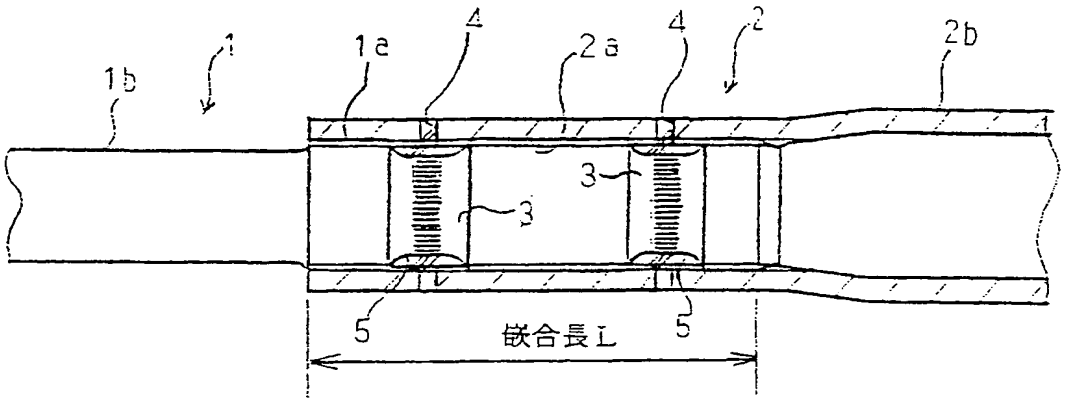


図 5

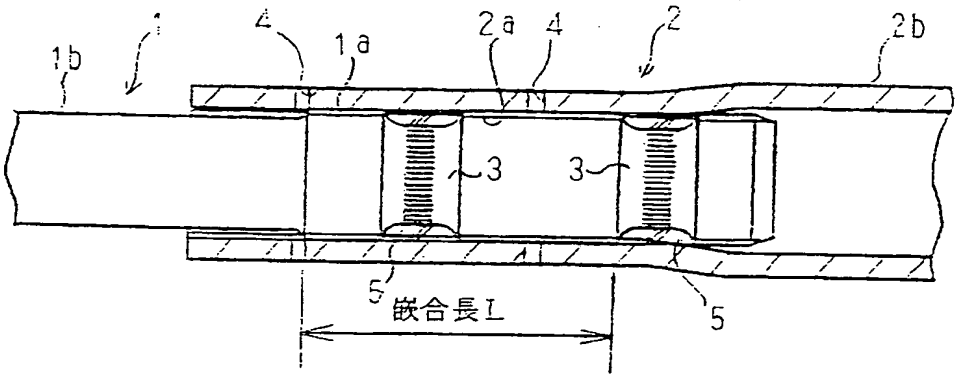
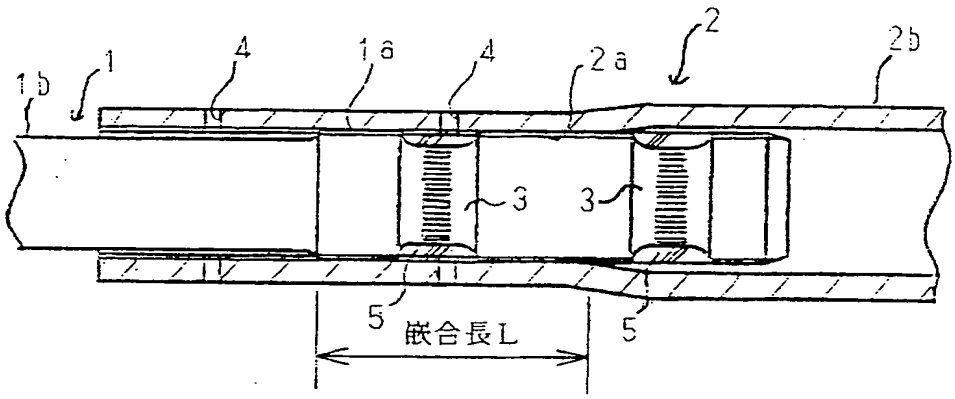


図 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/00532

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] F16D3/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] F16D3/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1926-1996 Toroku Jitsuyo Shinan Koho 1994-2001
Kokai Jitsuyo Shinan Koho 1971-2001 Jitsuyo Shinan Toroku Koho 1996-2001

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GB, 002316150, A (Kabushiki Kaisha Yamada Seisakusho), 18 February, 1998 (18.02.98), Fig. 8, & JP, 10-45006, A (Yamada Seisakusho et al.)	1,2
Y	JP, 5-185511, A (Gunze Limited), 27 July, 1993 (27.07.1993), Fig. 2; page 1, left column, lines 32 to 37, (Family: none)	1,2
Y	JP, 10-246369, A (Bridgestone Corporation), 14 September, 1998 (14.09.98), Fig. 1; page 2, right column, lines 5 to 31, (Family: none)	2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search
04 April, 2001 (04.04.01)

Date of mailing of the international search report
17 April, 2001 (17.04.01)

Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

国際調査報告

国際出願番号　PCT／JP01／00532

A.　発明の属する分野の分類（国際特許分類（IPC））
Int.Cl.⁷　F16D3/06

B.　調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））
Int.Cl.⁷　F16D3/06

最小限資料以外の資料で調査を行った分野に含まれるもの
日本国実用新案公報　1926－1996年
日本国公開実用新案公報　1971－2001年
日本国登録実用新案公報　1994－2001年
日本国実用新案登録公報　1996－2001年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

C.　関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
Y	GB、002316150 、A (Kabushiki Kaisya Yamada Seisakusho) 18.2月.1998　(18.02.98) 、 Fig8、 & JP,10-45006,A (株式会社山田製作所他)	1、2
Y	JP,5-185511,A (グンゼ株式会社)、 27.7月、1993　(27.07.1993) 、 図2、第1ページ左欄第32行～同欄第37行、(ファミリーなし)	1、2

☒ C欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

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「O」口頭による開示、使用、展示等に言及する文献
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「&」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
04.04.01	17.04.01

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）		
日本国特許庁（ISA／JP） 郵便番号100－8915 東京都千代田区霞が関三丁目4番3号	仁木　浩　印	3J	8011
	電話番号　03－3581－1101　内線　3328		

様式PCT／ISA／210（第2ページ）（1998年7月）